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PERSPECTIVE

The stimulating adventure of KRN 7000†

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Associated with the CD1d protein, KRN 7000, a potent synthetic α-galactosylceramide, is known to activate the invariant NKT immune cells. This stimulation then leads to the production of different cytokines modulating a T_H1/T_H2 immune response balance involved in protection against several pathologies such as autoimmune diseases and cancers. Various efforts have been made toward the synthesis of simple and more functionalized analogues in order to selectively induce T_H1 or T_H2-type cytokine production. Since the discovery of KRN 7000, structure-activity relationships, crystallographic and modelling studies have pointed to the potential of several GalCer analogues in term of selective bioactivity, and have highlighted interesting elements in order to better understand the recognition and activation mechanisms of immune *i*NKT cells. By presenting an up-to-date library of analogues, collecting recent breakthroughs done in crystallography and molecular modelling, and relating them to the available biological results, we hope that this review will highlight and help the scientific community in their KRN research.

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1. Introduction

Nature abounds with an amazing amount of potentially bioactive compounds and has always aroused the interest of scientists. Marine invertebrates are known to be an important reservoir of such molecules.[1] At the present time, few drugs are actually derived from marine organisms, but several promising molecules are currently in preclinical/clinical trials.[2] Sponges, the simplest and most ancient multicellular animals on Earth, are the principal source of diverse bioactive cerebrosides. The

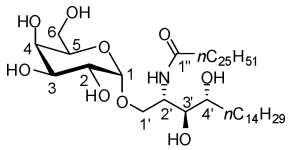
Aline Banchet-Cadeddu

Aline Banchet-Cadeddu was born in 1981 in France. In 2007, she received her PhD degree from the University of Reims, France, under the supervision of Prof. A. Haudrechy. Her research mainly concerned the development of a new synthetic strategy to prepare C*-glycoside skeletons, and the synthesis of an ester analogue of KRN 7000. In 2008, she joined the group of Prof. A. G. M. Barrett as a postdoctoral fellow at Imperial College, London (England) working on medicinal chemistry projects for Cancer Research UK. In 2009–2010, she was a science teacher at the French High School in London.*

Eric Hénon

After receiving his MSc in the group of Prof. Rivail (Nancy University), Eric Hénon obtained his PhD in theoretical chemistry in 1997 from University of Reims Champagne-Ardenne. His research was carried out in atmospheric chemistry in the group of Prof. Jacon where he investigated reaction mechanisms using kinetic theories (TST, VTST, RRKM) combined to quantum chemistry to calculate reaction rates. He was appointed full professor in 2008. Since he joined the Institut de Chimie Moléculaire de Reims, his research has been focusing on Organic/Bioorganic chemistry problems.

cerebrosides belong to the family of glycosphingolipids and are important components of a wide variety of tissues, organs and nerve cell membranes in biological systems.[3] They consist of a ceramide part (with two long fatty chains) linked to a polar residue (single sugar or polysaccharide). The gluco- and galactoceramides are the most well known members of this family and for almost two decades, one of them, KRN 7000 **1**, has shown promising bioactivities against diverse pathologies.[4] This compound is a synthetic glycolipid resulting from structure-activity relationship studies performed on glycolipid extracts of the Japanese marine sponge, *Agelas mauritianus* (Fig. 1).[5] Associated with the CD1d protein, this α-galactosylceramide (α-GalCer) interacts with one component of the immune system, the Natural Killer T (NKT) cells, and allows the activation of signalling molecules involved in cellular communication called cytokines. According to the nature of the produced cytokines, a T_H1/T_H2 immune response profile can thus be drawn up and involves the activation of certain other immune cells (B cells, T cells, macrophages...), to fight against tumours and antimicrobial functions (T_H1) or to protect against autoimmune diseases (T_H2).[4]



1 KRN 7000

Fig. 1 KRN 7000, a synthetic bioactive glycolipid.

Since its discovery, various efforts have been made toward the synthesis of simple and more functionalised analogues in order to selectively induce T_H1 or T_H2-type cytokine production and to better understand the interaction mechanism of the CD1d/Glycolipid/NKT complex.

Manuel Dauchez

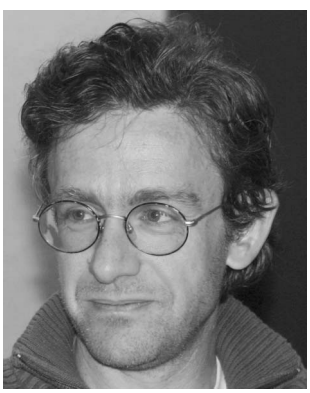
Manuel Dauchez was born in 1961. He graduated in physics at the University of Amiens in 1983. He then moved to the University of Lille 1 where he became PhD in 1990, in Prof. G. Vergoten's Lab, working on spectroscopic force fields of carbohydrates. In 1991, he spent a post-doc period at the University of Oregon, under the supervision of Prof. W. Peticolas and then became assistant professor in 1993 at the University of Reims. He is now full professor at the department of biochemistry using molecular modelling approaches and developing specific tools to study proteins, peptides and glycolipids.

Jean-Hugues Renault

Jean-Hugues Renault was born in 1968 and received his PhD degree in the field of Phytochemistry and Separation Sciences from the French University of Reims Champagne-Ardenne in 1997. He is now full professor of pharmacognosy at the University of Reims Champagne-Ardenne (Institut of Molecular Chemistry of Reims, UMR CNRS 6229). His research activities are devoted to the development of separation processes using support-free liquid–liquid chromatographic techniques, such as centrifugal partition chromatography or centrifugal partition extraction. Besides methodological developments, the applications essentially concern the purification of natural or synthetic organic compounds.

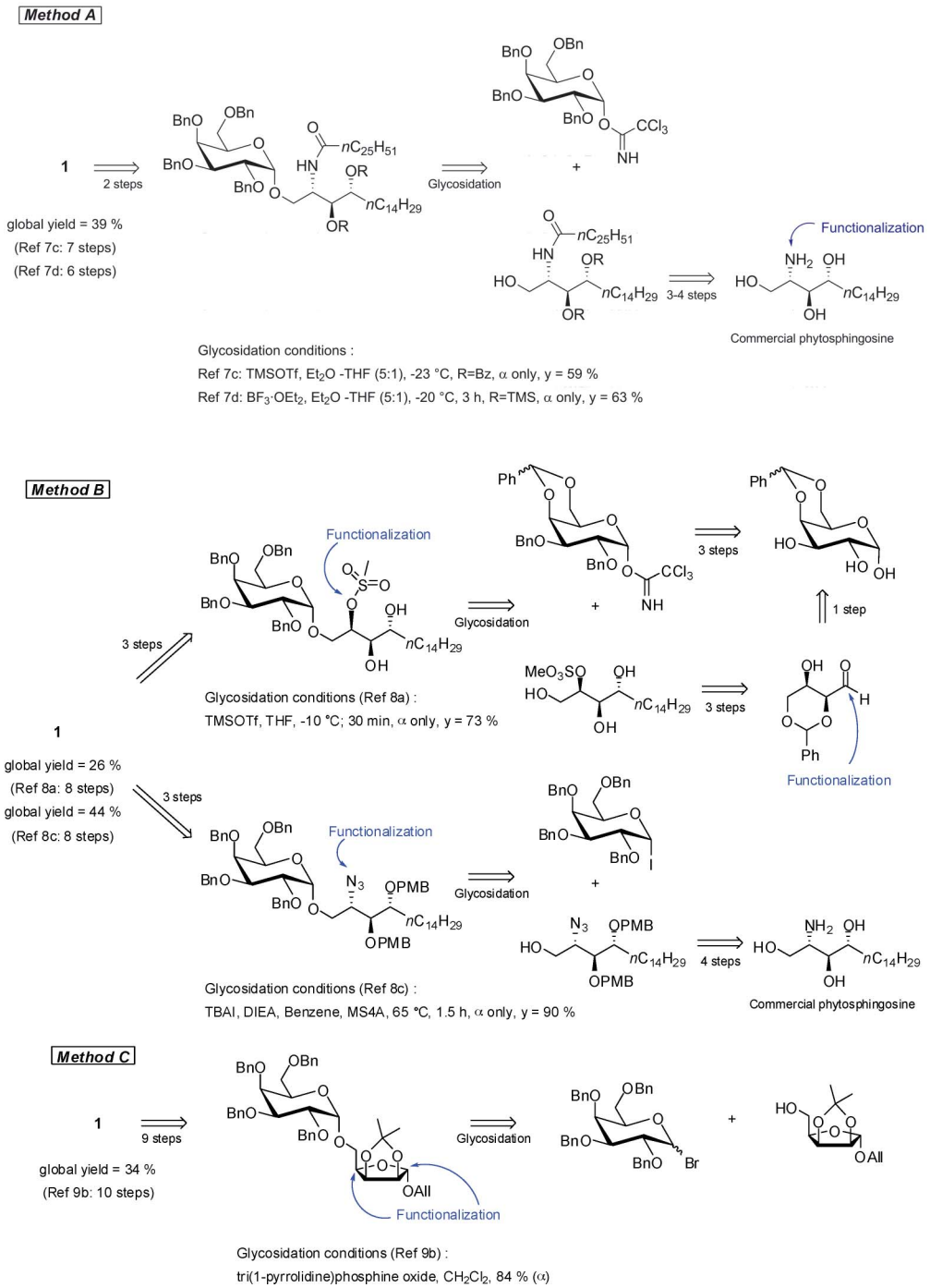
Fanny Monneaux

Fanny Monneaux received her PhD from University of Paris V (2001). After two post-doctoral positions, at the Institut de Biologie Moléculaire et Cellulaire, CNRS with Dr Sylviane Muller (Strasbourg) and in the INSERM U 561 laboratory with Prof. Christian Boitard (Paris), she began her academic career as a CNRS researcher in 2004. Her current research is focused on the study of molecular and cellular mechanisms involved in systemic autoimmune diseases (systemic lupus erythematosus and rheumatoid arthritis), and more specifically on the analysis of regulatory T cell numbers and functions ($CD4^+CD25^+$ and iNKT cells) in patients and autoimmune mice.



Arnaud Haudrechy

Arnaud Haudrechy obtained his PhD degree in 1990 from the Paris VI (with Prof. P. Sinaÿ) in the field of carbohydrates and transition-metal chemistry (Tebbes reagent). Then he joined Professor Y. Kishi (Harvard University) as a postdoctoral fellow working on preferred conformations of C-glycosides. In 1992, he joined the University Paris Sud to work, with Prof. Y. Langlois, on low-temperature asymmetric Diels–Alder reactions and sequential Claisen–Ireland/metathesis concept. He is now full professor of organic chemistry at the University of Reims Champagne-Ardenne, and his research program is focused on the use of carbohydrates for the synthesis of biologically active molecules.



Scheme 1 Major pathways for the synthesis of KRN 7000.

2. An interesting immunostimulating compound: KRN 7000

2.1. Syntheses of KRN 7000

This α-GalCer is the patented property of Kirin Brewery Co.[6] Its limited commercial availability and high price has led to the publication of many different syntheses. These can be classified into three categories: 1) total functionalization of the ceramide part before coupling with the sugar (method A),[6,7] 2) *N*-acylation after the glycosidation step (method B),[8] 3) insertion of both fatty chains after the coupling (method C)[9] (Scheme 1). In these three ways, the major difficulty is the formation of the glycoside bond with high α stereoselectivity. Several methods have been reported in the literature and the results depend upon the donor/acceptor character of the reagents (both the sugar and ceramide precursors).[7,8,10] Indeed, the nature of the protecting groups (benzoyl, TBS, benzyl) and the choice of the amide precursor (azide, protected alcohol, amide) greatly influences the nucleophilicity of the sphingosine part (acceptor character). For

example, Xia *et al.* have shown that when the alcohols on the sugar are protected with benzyl groups and the amide function is already inserted, only the β configuration is obtained. Protection with benzoyl groups leads to the expected α anomer, under the same conditions.[7d] Low nucleophilicity of the ceramide precursor is thus recommended and trichloroacetimidate or halogen (fluoride, iodide) α-galactoside donors are usually preferred. Basically, the first two convergent synthetic pathways are the most convenient and, of course, the most widely used. But they often require expensive starting materials like phytosphingosines[7] or long syntheses to obtain the ceramide.[6] This last point can be improved as seen in the efficient strategy recently developed by Castillón and co-workers.[11] Moreover, easy access to a library of analogue compounds is limited with method A and partial with pathway B. The C strategy, developed by Ito and Tsujimoto,[9a] is admittedly less convergent and leads to KRN 7000 with a low overall yield, but inexpensive starting material is used (tartaric ester), and the formation of an azidothreitol as a common intermediate (yield = 17%) is advantageous to easily diversify the ceramide part. In order to gain several steps, Panza and co-workers have cleverly chosen to form a disaccharide as a common precursor (yield = 84%) followed by opening of a furanose moiety for the insertion of both fatty chains (Scheme 1).[9b] Compound **1** was thus obtained in gram scale in 10 steps in 34% overall yield. This strategy has also been applied to the synthesis of diverse oxa-analogues (see Table 4).

2.2. Cellular recognition (CD1d/Glycolipid/TCR) and activity

The uniqueness of KRN 7000 is its wide range of activity.[4,12] Indeed, when it is administered early in the course of disease development, α-GalCer has shown potential in growth prevention or even the onset of several types of tumour cells (hepatitis, lung cells . . .),[7a,13] pathologies such as malaria,[14] tuberculosis,[15] fungal pathogens,[16] inflammation[17] and auto-immune diseases such as lupus[18] or diabetes.[19] KRN 7000 has also shown to be an effective adjuvant for vaccines (malaria, HIV . . .)[14b,20] and could be useful in preventing complications in grafts.[21] Other specific therapeutic strategies involving α-GalCer have shown encouraging results in the growth suppression of established tumours such as liver[22a] or melanoma[22b] metastases in mice. Moreover recent studies have shown it as a potential treatment in preventing the development of asthma.[23] It is worth mentioning that, *in vitro*, KRN 7000 is not directly cytotoxic against tumour cells. Actually, it has been shown that it specifically interacts with one cell category of the immune system, the Natural Killer T (NKT) cells.[24] Thanks to this property, KRN 7000 has become a useful tool in therapeutic research as a potential pharmacological agent and pharmacokinetic studies[25] as well as clinical trials have been started.[13h–i,26]

To generate an activity, two consecutive cellular recognitions are necessary. First of all, the α-GalCer is recognized by the CD1d protein. Then, the new binary complex interacts with the NKT cell receptor (TCR) to form the active ternary complex.

2.2.1. Binary complex. The CD1d protein is associated with a β2-microglobulin (β2m) and is carried by an antigen presenting cell (APC) such as dendritic cells or macrophages. The CD1d belongs to a five member family of large clusters of non-polymorphic, major histocompatibility complex (MHC) class-I-like molecules that bind distinct lipid-based antigens that are recognized by T cells.[27] Recently, the crystallographic data of the human-CD1d/α-GalCer complex was reported by Koch *et al.*[28] and Zajonc *et al.*[29] Useful information is now available: 1) a description of the basic architecture of human CD1d; 2) the position of the glycolipid inside the cavity of the protein (Fig. 2) and, above all, 3) this data has allowed the study of the stabilizing interactions between the ligand and its host. The CD1d is constituted by a *C*-terminal part, essentially composed of sheets in two layers, and an *N*-terminus domain, structurally more complex, characterized by two helices acting as a 'jaw' and overlapping a β-sheet floor. They differ from the MHC molecules by a big cavity containing two hydrophobic pockets, A' and C' (also called F' pocket), in the *N*-terminal part (Fig. 2). The glycolipid has a favoured orientation in the protein. On one hand, each long lipid chain is accommodated in one hydrophobic pocket. The acyl chain is hosted in the A' pocket and supported by the β-sheet floor, and the sphingosine chain is in the C' pocket. The lipid chains are thus stabilized by hydrophobic interactions with amino acids from the β-sheet floor and helices. Certain residues such as tryptophans and Cys12 may certainly play a role in the insertion of the long acyl chain into the protein and its adaptation to the shape of the pocket. On the other hand, the sugar is pinned down between both helices, and emerges from the protein, ready to interact with the TCR (Fig. 2). Suggested by the X-ray structure data, the ligand is also stabilized with hydrogen bonds (Fig. 3). The first interaction involves the amino acid Asp151 (belonging to the α2-helix) and two hydroxyl groups on D-Galactose (2-OH and 3-OH). The Thr154 (α2-helix) acts as an H-bond donor with Asp151 and as an H-bond acceptor with the amide group. This resulting H-bond network was called OTAN (2-***O***H, ***T***hr154, ***A***sp151, 2′-***N***H) by Hénon *et al.*[30] Finally, one hydroxyl group (3′-OH) of the phytosphingosine chain is hydrogen bonded to the Asp80 (α1-helix). It should be noted that this 3′-OH group also points towards the outside of the groove. Recently, molecular dynamic (MD) simulations performed in our laboratory have allowed us to monitor the behaviour of the polar head during the modelling.[30] We have shown that the strong H-bonds between the hydroxyl functions in positions 2 and 3 on the sugar and the Asp151 prevent the head from rotating and hold the 4-OH group in a specific position. Furthermore, the tryptophan Trp153 on the α2-helix has a good affinity (van der Waals contact) with the "least hydrophilic" side of the D-Galactose and also takes part in the orientation of the polar head (Fig. 4). It is worth noting that, during our MD simulations, no hydrogen bond between the anomeric oxygen and an amino acid residue from the helices was observed. This result supports the idea that the *C*-glycoside analogues may have similar binding properties with CD1d. However, as suggested by X. Li *et al.*, the O/CH_2 group switch may alter the interaction in the ternary complex.[31] Concerning the sphingosine part, our MD studies have shown that the 4′-OH group is eventually involved in the strong H-bond network with Asp80 (Fig. 3). KRN 7000 is thus well stabilized in the protein and the complex CD1d/α-GalCer can interact with the NKT cells receptor.

2.2.2. Ternary complex. NKT cells, a subpopulation of the T lymphocyte family, are involved in a broad range of diseases and represent an important immunotherapeutic target with large clinical potential.[32] NKT cells differ from the conventional

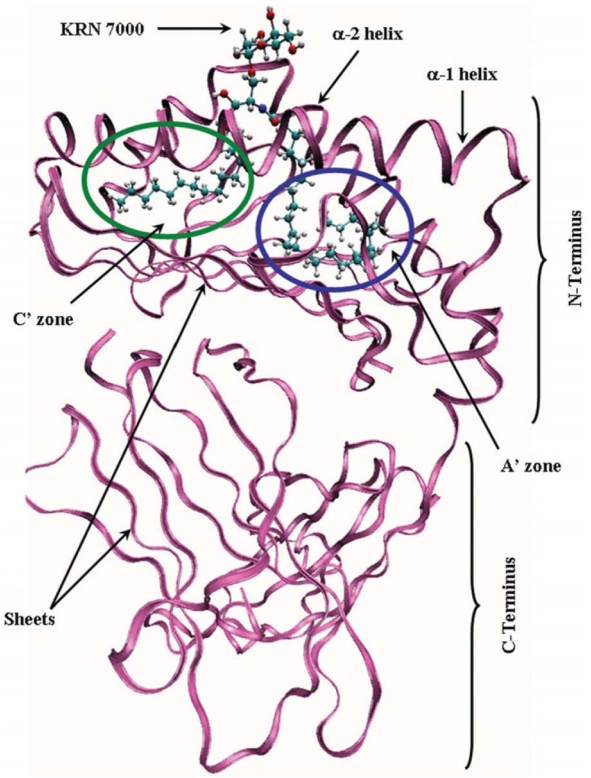


Fig. 2 The antigen and its host.

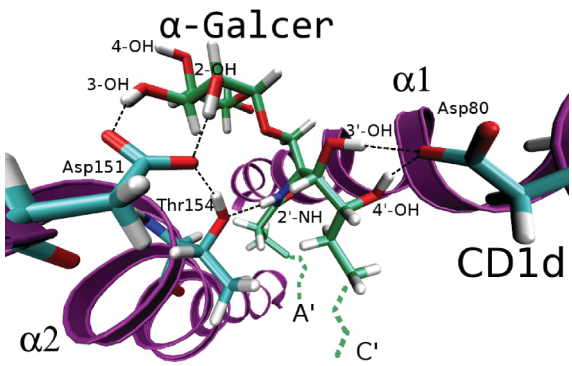


Fig. 3 Hydrogen bond (OTAN) network in the binary complex.

cytotoxic T_C lymphocytes recognizable by a CD8 marker and helper T_H lymphocytes possessing a CD4 marker. On their surface, they express both CD3/T cell antigen receptor (TCR) and some *Natural Killer* (NK) cell characteristics such as an NKR P-1 receptor (called NK1.1 in mice).[33] The TCR is a heterodimer consisting of α and β polypeptide chains. Each chain is made up of a variable N-terminus domain, called V, and an unchanging C-terminal part, noted C. Two types of TCR can thus be distinguished: a semi-invariant TCR (*inv*TCR or *i*NKT), possessing an invariant chain Vα24-Jα18 often associated with the Vβ11 gene (Vα14-Jα18 and Vβ8 in mice) and a universal TCR, also called diversified TCR.[34] The *inv*TCR is common and specifically recognizes the binary CD1d/glycolipid complex.[35] The recent crystal structure of the ternary complex human-CD1d/α-GalCer/human-TCR published by Borg *et al.*[36] is a breakthrough in understanding the mechanism of the TCR interactions with the CD1d/antigen complex. In particular, three

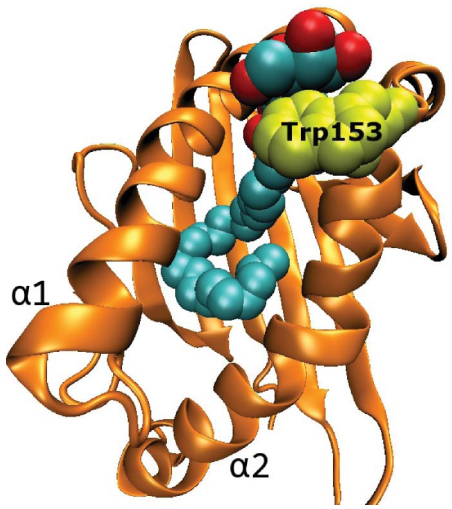


Fig. 4 Trp153 is very close to the least hydrophilic side of the D-galactose.

CDRs (Complementarity Determining Regions) present on the Vα and Vβ chains are implicated in recognition. TCR binding is dominated by the contacts between the α1 CD1d helix and these regions. The CDR1α interacts solely with the 3-OH and the 4-OH on the sugar *via* two hydrogen bonds. As can be seen from Fig. 5, the CDR3α loop (Jα18 section) is the most important recognition element, coming into contact with the sphingosine chain and both CD1d helices. It straddles the groove and stabilizes both helices and the two hydroxyl groups (2-OH on D-Galactose and 3′-OH on the phytosphingosine chain) thanks to a strong H-bond network (Table 1, Fig. 5). Although less involved, CDR2β and CDR3β interact with α1 and α2 helices, respectively. The involvement of the 3′-OH in binding with TCR supports the data of the SAR studies showing the importance of this function to observe an activity (see Table 3). According to a recent computational SAR study on the ternary complex performed by Nadas *et al.*,[37] Glu83 and Asp80 are the two most important residues on CD1d allowing for recognition and binding of TCR. Indeed, with the help of prior mutation studies performed by Rossjohn and co-workers,[38] Nadas *et al.* have thus shown that the lack of Asp80, Arg95 or Glu83 has a major impact on the binding affinity with TCR, whereas the lack of Asp94 and Ser30 is less notable. All these results show us that the orientation of the glycolipid before presentation to the TCR is decisive and suggests a lock-and-key interaction.[36,39]

Finally, recognition also involves other markers present on the NKT cell. With help from surrounding cytokines, they identify which kind of APC carries the binary complex. The nature of the APC depends on the organ where the recognition takes place (liver, spleen . . .).[40]

2.2.3. Cytokine production. The efficient binding of the CD1d/glycolipid/TCR allows the production of cytokines. These are communicating protein-type factors (possibly glycosylated), synthesized by the immune cells or by other cells or tissues acting remotely on diverse cells to regulate their activity or their function. With hormones and neuromediators, they are essential molecules involved in cellular interaction. Their secretion is brief, over a short distance, and is caused by the contact of an immune cell with an antigen *via* a specific receptor or another cytokine. When a cytokine is linked to its receptor, a cascade of metabolic

Table 1 Binary complex–TCR contacts

Binary complex\TCR	CDR1α	CDR3α	CDR2β	CDR3β
D-Galactose (KRN 7000)	Ser30α/3-OH Phe29α[a]/4-OH	Gly96/2-OH		
Sphingosine chain		Arg95α/3′-OH		
α1 (CD1d)		Asp94α/Arg79 Arg95α/Ser76, Arg79, Asp80 Leu99α/Phe84 Arg103α/Arg79	Tyr48β/Glu83, Lys86 Tyr50β/Glu83, Met87 Asn53β/Arg89	
α2 (CD1d)		Leu99α/Val147 Thr98α/Gln150		Tyr103β/Gln150

[a] carbonyl function of the Phe29α backbone.

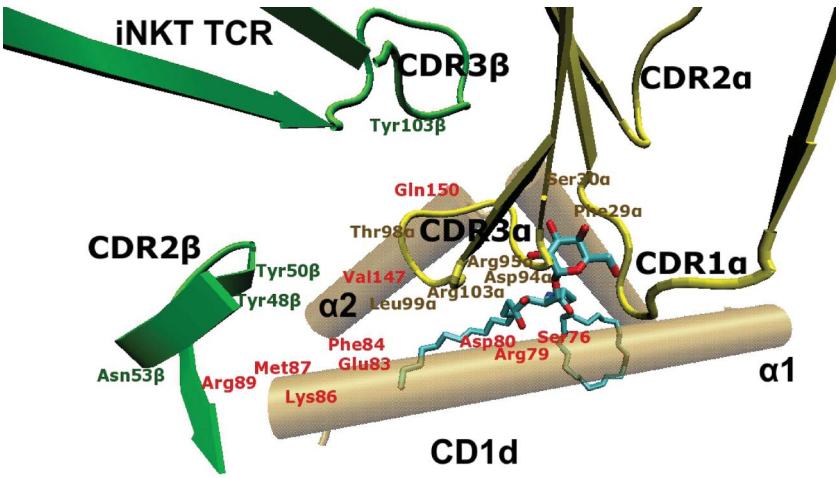


Fig. 5 Residues involved in the ternary complex.[30]

events occurs inside the cell (enzyme activation, expression of messenger RNA . . .) and involves the activation or the modification of certain cells. There are several families of cytokines: interleukins (IL), interferons (IFN-α, -β, -γ), chemokines, tumours necrosis factors (TNF), colony stimulating factors (CSF), etc . . . According to their biological activities, they are classified in three categories. The pro-inflammatory cytokines (IFN-γ, IL-2, IL-3, TNF-β, GM-CSF . . .) characterize a T_H1 (T Helper) type response involving cell-mediated immunity such as the activation of macrophages,[41] NK cells, antigen-specific cytotoxic T-lymphocytes to fight against tumours, or viral/bacterial/parasitic infections. The immunomodulatory cytokines (IL-4, IL-5, IL-10, IL-13 . . .) lead to a T_H2 phenotype specific of the humoral immunity with the activation of lymphocytes B[42] and the production of immunoglobulins and antibodies. The production of such immunomodulatory cytokines with regulatory function is desired for the treatment of autoimmune diseases. The effector cytokines (IFN, TNF . . .) provide defence against infectious agents and cancers. Cytokines thus spark off a complex network of relationships between all cells involved in the immune defence system. Moreover, cytokines have been recently used as therapeutic agents (G-CSF to facilitate the hematological reconstruction . . .) or targets (TNF in Crohn disease, rheumatoid polyarthritis . . .).[43] The recognition of the binary complex CD1d/KRN 7000 by the TCR promotes the rapid secretion of cytokines such as IFN-γ and IL-4 by the NKT cells and IL-12 by the antigen presenting cell, and supports a mild T_H1 response (Fig. 6).[35b,44] However, how can the NKT cells induce an efficient immune activity by producing T_H1 and T_H2 type cytokines which have opposing biological effects?[45] Indeed, on one hand, IFN-γ and IL-12 inhibits a T_H2 phenotype, and on the other hand, IL-4 deactivates a T_H1 one whereas IL-12 activates it. Apparently, the biological behaviour of KRN 7000 is time dependant. Although NKT cells clearly produce detectable levels of both IL-4 and IFN-γ within 2 h of stimulation, they switch to a T_H1-like cytokine profile by 16 h, which continues for at least 3 days after this antigenic challenge.[44] The cytokine profile shift is also determined by the quality of the signal delivered by the TCR to the *i*NKT. Among the factors that could cause this shift, the stability of the CD1d/glycolipid complex may play a significant role.[46] A less stable association between the glycolipid and CD1d could result in a shorter half-life for NKT cell stimulation[46a] and a fast dissociation of the binary complex.[46b] Another point to take into consideration is that the nature of the APC carrying the Glycolipid/CD1d complex also seems to have its importance in the profile of generated cytokines. Primary B cells such as APC elicit the production of IL-4 by the NKT, whereas dendritic cells (DC) have an impact on the secretion of IFN-γ.[40,47] The use of KRN 7000 for clinical therapy has not yet been successful due to the cytokines antagonism effect,[26] as T_H1- and T_H2-type cytokines can antagonize each other's biological functions. The concomitant secretion of both IFN-γ and IL-4 also limits the therapeutic potential of α-GalCer in autoimmunity, for example.

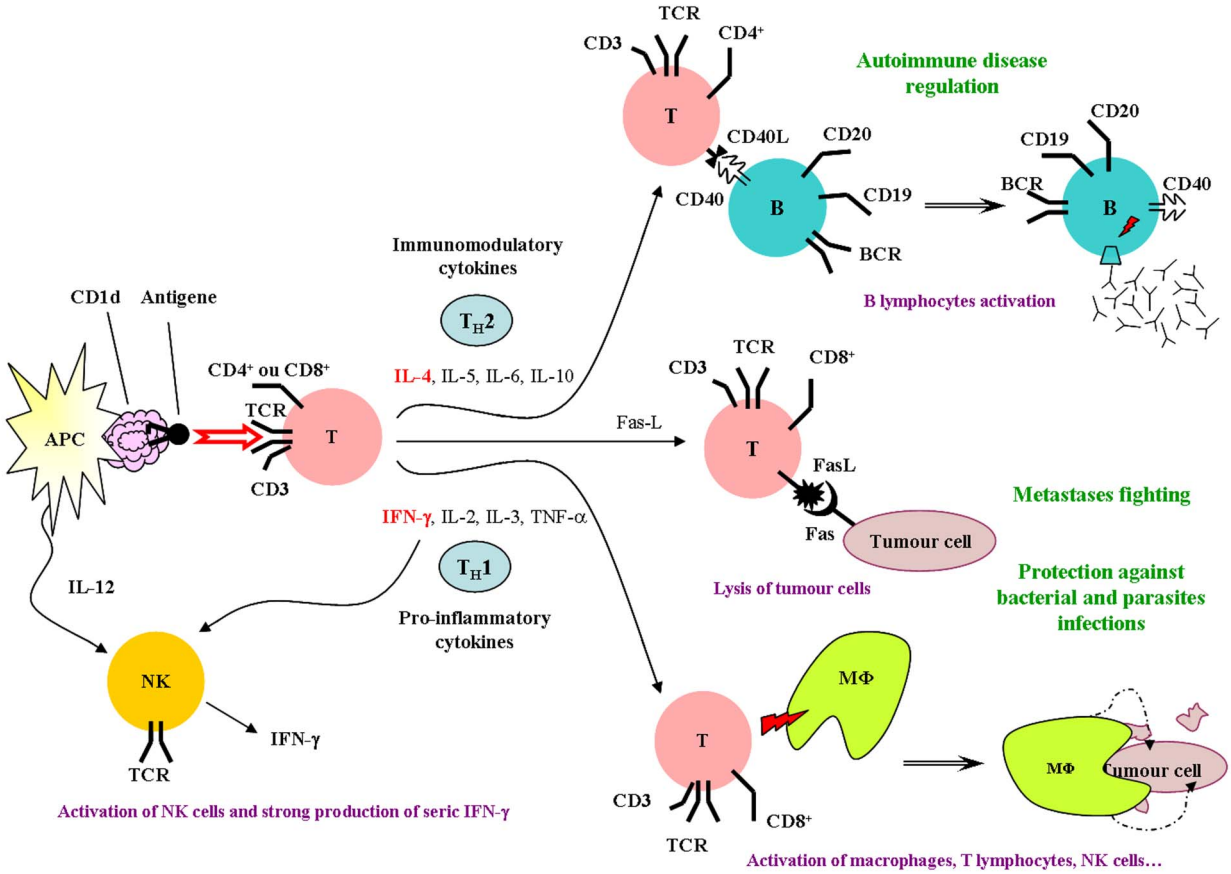


Fig. 6 Activation of the immune cells after recognition of the glycolipid by the TCR.

Moreover, the acquisition of an anergic phenotype by *i*NKT cells may also affect the efficiency of KRN 7000 administration. Indeed, recent investigations have shown that *i*NKT cells can become unresponsive to a second intravenous injection of the glycolipid whereas no loss of cytokine secretion was reported upon intradermal administration.[48]

To circumvent the problem, many research groups are trying to develop new analogues of KRN 7000, which could selectively induce the production of T_H1 or T_H2 type cytokines by the NKT cells.[49]

2.2.4. Glycolipid insertion into the CD1d. In addition to the binding features in the ternary complex, another essential question remains unanswered: how does the glycolipid enter the protein pocket? Molecular dynamics studies are currently in progress in our laboratory to understand the loading of the long lipid chains through the small entry to the cavity. Amazingly, preliminary results from a normal mode analysis have shown a jaw type movement, essentially characterized by the large amplitude of the α2-helix (Fig. 7).[50] Nadas *et al.* also observed that, in the absence of the ligand, the A' pocket seemed to be closed whereas the C' pocket remained quite accessible and only latched with the Trp153 residue.[37] They hypothesized that the ligand could be loaded into the CD1d binding groove by entering the C' pocket, thus causing the slow opening of the A' pocket to accommodate the ligand. They also suggested that an acyl chain or

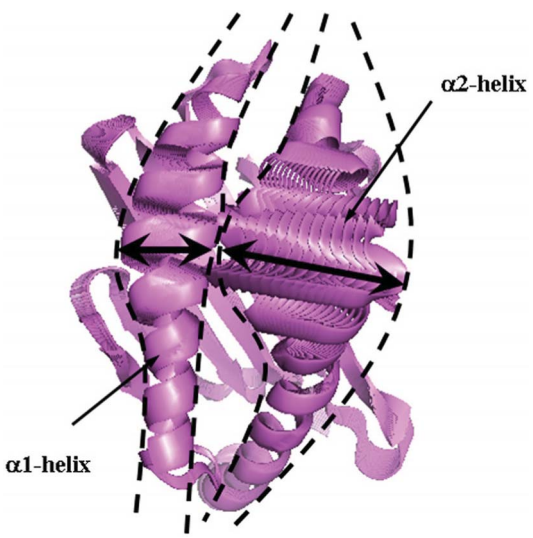


Fig. 7 Jaw type movement of the CD1d to load the glycolipid.

spacer molecule of appropriate length[51] is required to provide the optimum orientation of important amino acid residues to form the necessary contacts with TCR. Further MD studies are still necessary to better understand this loading mechanism and the use of steered dynamics could be of help to simulate glycolipid

removal from the pocket and thus define the exit trajectory of the ligand.

According to kinetic studies, αGalCer loading into mouse CD1d is very slow. Moreover, the dissociation of the lipid-CD1d complex is remarkably slow with a half-life in excess of 1 day.[39b] On the other hand, a recent study has shown that T_H2 cell-type-biasing glycolipids with relatively short or polyunsaturated alkyl tails (see 3.1.2.b) are presented to CD1d with rapid kinetics and without help from detergent or lipid transfer proteins (such as saposin B). The physiological pH is also a major factor for good stability of the binary complex. Recombinant saposins have indeed the ability to unload short glycolipids from CD1d at pH 5.0, but not at neutral pH.[52]

3. Analogues of KRN 7000

The library of KRN 7000 analogues is based on four types of modifications: substitution and variation of the sugar; modification of the polar portion of the ceramide; variation on the lipid chains; and modification of the configuration and the nature of the glycoside bond (Fig. 8).

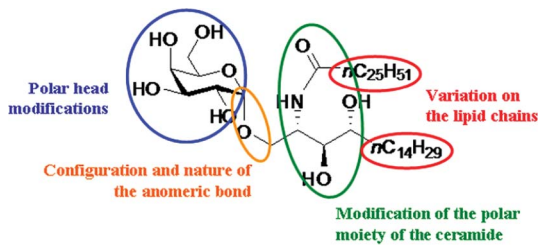


Fig. 8 Four types of modifications to access KRN 7000 analogues.

3.1. *O*-glycoside analogues

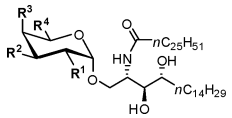
3.1.1. Modification and substitution of the sugar. To determine the influence of the sugar subset, SAR studies have shown that α-D-galactosylceramide is more potent than the α-D-glucosylceramide, itself more active than the α-D-mannosylceramide.[39a,53] This result is in line with the restriction of CD1d for D-galactosyl- and D-glucosyl compounds. The D-mannosyl form is preferentially recognized by the CD1b. The importance of the hydroxyl functions in positions 2, 3 and 4 has been tested by suppression of the hydroxyl group (**8**, **9**)[54] and by substitution with fluoro (**10–12**),[54,55] methoxy (**13**)[56] or sulfate (**14**)[57] groups (Table 2). Capping or substituting the hydroxyl group in position 2 gives totally inactive compounds (**10**, **13**), whereas the 3- and 4-deoxy and -fluorogalactosyl derivatives (**8**, **9**, **11** and **12**) still stimulate some NKT cell hydridomas based on levels of IL-2 production. The 3-*O*-sulfate compound (**14**) gives a similar *i*NKT cell stimulation compared to KRN 7000 by keeping the H-bond network in place and enough contacts with the TCR. Moreover, no loss in activity is observed when the D-galactose moiety is substituted with other sugars in the C2, C4 and C6 positions (**15–17**) (Table 2) thanks to *in vivo* cleavage of the disaccharide by APCs, except in the case of a disaccharide in position 6.[53,58] Apparently, the interaction with the TCR seems to tolerate a small molecule in this position. Indeed, according to the crystal structure of the ternary complex, there is space between the active areas CDR1α and CDR3α of the TCR. New "C6-analogues" have thus been synthesized (Table 2): an amide series (fluorophore (**18–20**), biotin (**21**),[59] acetylamide (**22**, **23**)[60] and electron withdrawing substituents on an aryl ring (**24**)[61]); acid (**25**)[62] and methoxyether derivatives (**26** called RCAI-61).[63] Most of them do not significantly increase the ability of the glycolipids to stimulate T cells and are used to study interaction and loading of the glycolipid in the CD1d. However the electron-withdrawing amide derivative (**24**), the acid (**25**) and RCAI-61 (**26**) induce quite a strong T_H1 bias. The proximity of the Trp153 to the 6-position might have an effect on cytokine polarisation thanks to a π-stacking with the electron rich indole ring and the aryl **24** or van der Waals interactions with the acid derivative (**25**).[30,64] The activity of RCAI-61 (**26**) has been explained by the authors to be due to an increase in the electron density of the 4-OH group which is no longer hydrogen bonded with the capped 6-OH and readily available for TCR recognition. The presence of the methyl group favours a good van der Waals contact with the Trp153, and the well known steric hindrance of this type of substituent could also have an impact on the overall stabilization of the ternary complex. This last point might be related to the production of a large amount of IL-12, mainly causing *in vivo* IFN-γ production in mice and involving the inhibition of released IL-4 (see 2.2.3.). A slight increase of the production of IL-12 has also been observed with acid derivative **25**. These observations should be taken into consideration when planning the synthesis of new promising T_H1 analogues. Finally, in order to improve the bioavailability of the α-GalCer, a water-soluble derivative (**27**) has recently been prepared by introducing a methyl-PEG substituent through an amide bond in position 6 of the sugar (Table 2).[65] It exhibits improved physical and biological properties, and also retains both the specificity for the CD1d receptor and the stimulatory properties on immune cells. This new synthetic derivative represents a promising tool for pharmacokinetic studies. More surprisingly, it is also the first analogue of this series to induce a T_H2 immune response. In the future, it would be interesting to study docking models of this compound in order to understand this activity reversal.

Molecular modelling studies have been performed by Nadas *et al*. in order to understand the effect of sugar modification on the ternary complex.[37] A library of analogues was built, where the sugar was replaced or substituted in positions C2, C3, or C4 as well as with a truncated acyl chain (nC_7H_{15}). Docking studies have shown that, as expected, the modifications in the C2 and C3 positions of the sugar are not tolerated by the tertiary complex. However, substitutions in the C4 position seem to be viable. Apparently, aliphatic amide, ester or ether groups present in this position maintain a similar orientation and hydrogen binding compared to α-GalCer. As, indeed, few C4-analogues have been synthesized and tested, it would be interesting to explore this point. It was also found that the α,β-glucoceramides and β-GalCer bound CD1d better than α-GalCer. However, the β anomers had a worse binding energy with TCR compared to the α derivatives, which is consistent with the experimental studies.

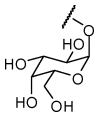
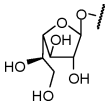
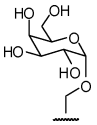
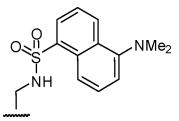
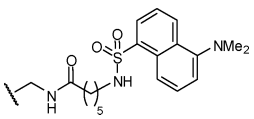
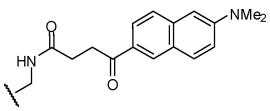
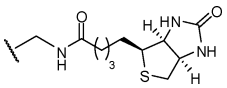
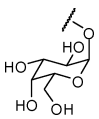
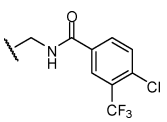
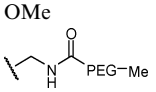
3.1.2. Modification of the ceramide moiety. The second major area of analogue research has been essentially focused on the modification of the ceramide.

3.1.2. a) Modification of the phytosphingosine polar part. Structural requirements of the ceramide for anti-tumour activity

Table 2 Substitution on the sugar moiety

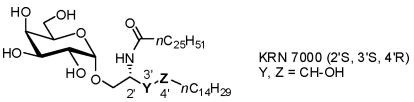


KRN 7000
R^1, R^2, R^3 = OH
R^4 = CH_2OH

Analogues	R^1	R^2	R^3	R^4	Activity	Ref.
8[a]	OH	H	OH	CH_2OH	*i*NKT cell activation on 1.2 and 2H4 hybridomas[m]	54
9[a]	OH	OH	H	CH_2OH	*i*NKT cell activation on 1.2 and 2H4 hybridomas[m]	54
10	F	OH	OH	CH_2OH	No *i*NKT cell activation[m]	55
11[a]	OH	F	OH	CH_2OH	*i*NKT cell activation on 1.2 and 2H4 hybridomas[m]	54
12[a]	OH	OH	F	CH_2OH	*i*NKT cell activation on 1.2, 2H4, 1.4 hybridomas[m]	54
13	OMe	OH	OH	CH_2OH	No *i*NKT cell activation[m]	56
14	OH	OSO_3Na	OH	CH_2OH	*i*NKT cell activation (→)[h]	57
15		OH	OH	CH_2OH	*i*NKT cell activation (→)[m,h]	53, 58
16	OH		OH	CH_2OH	*i*NKT cell activation (→)[m]	53, 58a
17	OH	OH	OH		*i*NKT cell activation (→)[m]	53, 58a
18	OH	OH	OH		*i*NKT cell activation (~↘)[m]	59
19	OH	OH	OH		*i*NKT cell activation (→)[m]	59
20	OH	OH	OH		*i*NKT cell activation (~↘)[m]	59
21	OH	OH	OH		*i*NKT cell activation (~↗)[m]	59
22 (PBS-57)[b]	OH	OH	OH		*i*NKT cell activation (→)[m+,h]	60a
23[c]		OH	OH		*i*NKT cell activation (↘)[m]	60b
24	OH	OH	OH		T_H1 (~↗)[m+]	61
25	OH	OH	OH	COOH	T_H1 (~↗)[m+,h]	62
26 (RCAI-61)	OH	OH	OH	OMe	T_H1 (↗↗)[m+]	63
27	OH	OH	OH		T_H2[m+]	65

↘ less potent than KRN 7000; ↗ more potent than KRN 7000; → similar activity compared to KRN 7000; ~↗ or ~↘ slight increasing/decreasing; [m] *in vitro* tests performed on mice cells; [m+] *in vivo* tests performed on mice cells; [h] *in vitro* tests performed on human cells; **NC**: not communicated. [a] Sphinganine analogues (4′-deoxy). [b] Chain acyl possessing a Z double bond: -$(CH_2)_{13}(CH{=}CH)C_8H_{17}$. [c] Truncated chain acyl nC_7H_{15}.

Table 3 Modification of the polar part of the ceramide moiety



Analogues	Configuration 2′	Y (configuration 3′)	Z (configuration 4′)	Activity	Ref.
28	*S*	CH_2	CH_2	No *i*NKT cell activation[m]	39a
29	*S*	CH–OH (*R*)	CH–OH (*R*)	*i*NKT cell activation (↗)[m],(↘)[h]	66
30	*S*	CH–OH (*R*)	CH–OH (*S*)	*i*NKT cell activation (↘↘)[m,h]	66
31	*S*	CH–OH (*S*)	CH–OH (*S*)	T_H1 (→)[m,h]	66
32	*R*	CH–OH (*R*)	CH–OH (*R*)	*i*NKT cell activation (↘↘)[m,h]	66
33	*R*	CH–OH (*R*)	CH–OH (*S*)	*i*NKT cell activation (↗)[m],(↘)[h]	66
34	*R*	CH–OH (*S*)	CH–OH (*R*)	No activity[m,h]	66
35	*R*	CH–OH (*S*)	CH–OH (*S*)	*i*NKT cell activation (↘)[m,h]	66
36[a]	*S*	CH–OH (*S*)	CH–OH (*S*)	T_H1 (→)[m]	67
37	*S*	CH–OH (*R*)	CH_2	*i*NKT cell activation (→)[m, m+] T_H1 (→)[h]	39a, 68a,68c 68a,68b
38[b]	*S*	CH–OH (*R*)	CH_2	High tumour growth inhibition[m+]	69
39[b]	*S*	CH–OH (*S*)	CH_2	Weak tumour growth inhibition[m+]	69
40[b]	*R*	CH–OH (*R*)	CH_2	Weak tumour growth inhibition[m+]	69
41[b]	*R*	CH–OH (*S*)	CH_2	Weak tumour growth inhibition[m+]	69
42	*S*	CH–OH (*S*)	CF_2	T_H1[m+]	70
43	*S*	CH–NH_2 (*S*)	CH–OH (*S*)	T_H1 (↘↘)[m+]	71
44	*S*	CH–NH_2 (*R*)	CH–OH (*S*)	T_H1 (↘↘)[m+]	71
45	*S*	CH–OH (*S*)	CH–NH_2 (*S*)	T_H1 (↘)[m+]	71
46	*S*	CH–OH (*S*)	CH–NH_2 (*R*)	T_H1 (↘)[m+]	71

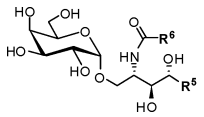
↘ Less potent than KRN 7000; ↗ more potent than KRN 7000; → similar activity compared to KRN 7000; ~↗ or ~↘ slight increasing/decreasing; [m] *in vitro* tests performed on mice cells; [m+] *in vivo* tests performed on mice cells; [h] *in vitro* tests performed on human cells; **NC**: not communicated. [a] Truncated acyl chain nC_7H_{15}. [b] Truncated acyl and sphingosine chains $nC_{13}H_{27}$.

have been demonstrated. No activity is detected when both hydroxyl groups in the 3′ and 4′ positions are removed (**28**, Table 3).[39a] This loss of activity is not surprising now, knowing the involvement of the 3′-OH in the recognition with the TCR according to the crystal structure.[36] Recently, the importance of the amide configuration and the diol on the ceramide part was highlighted by Park *et al.*[66] After achieving the synthesis of several KRN 7000 stereoisomers (**29–35**) (Table 3), biological studies have revealed the crucial importance of the *S* configuration of the amide and the hydroxyl group in position 3′ to induce activity. Changing the stereochemistry of the second hydroxyl group (4′-OH) did not show any improvement in the production of cytokines. *In vitro* tests on mice *i*NKT cells, also performed on the truncated stereoisomer **36** by Trappeniers *et al.*, have shown a similar activity to KRN 7000.[67] The 4′-deoxy analogue (**37**) is nearly as active as KRN 7000 in stimulating human and mice NKT cells.[39a,68] However its poor solubility compared to the parent compound during biological tests might limit its use as a potential candidate for further studies.[68a] These results are in line with the previous observations done with sphinganine analogues (**38–41**) (Table 3), for which the hydroxyl function in position 4′ is missing.[69] In 2008, Linclau and co-workers investigated the effect of the substitution of the 4′ position with a CF_2 function (**42**) (Table 3).[70] The presence of a difluoro group improves the hydrogen bond donating capacity of the 3′-OH with the Asp80 on the CD1d, but significantly reduces its ability to accept a hydrogen bond with Arg95 from the NKT TCR. The authors suggest that the interaction involving Arg95 on TCR is less important than the involvement of other residues in recognition. According to previous modelling studies,[37,38] this result rather seems to indicate that the Asp80/Arg95 and 3′-OH/Asp80 bindings are both probably reinforced to compensate for the weak link between the 3′-OH and Arg95, and thus still induce a decent T_H1 activity in this case.

Trappeniers *et al.* have recently studied the modification of certain elements directly involved in recognition with TCR, by replacing the hydroxyl groups in positions 3′ and 4′ by an amine function.[71] On one hand, the insertion of an amine group instead of the 3′-OH (compounds **43** and **44**) gave a dramatic drop in the production of cytokines compared to KRN 7000 despite a quite good affinity for the TCR, regardless the stereochemistry. Apparently, in this case, the stability of the ternary complex was well assured by the usual residues but the inability of the 3′-NH_2 to form an H-bond with Arg95 was decisive in term of activity. It is worth noting that at physiological pH, the amine could be protonated and positively charged, hence it would repel Arg95 which is likewise positively charged. On the other hand, the 4′-amino epimers (**45** and **46**) tended to induce a moderate activity, still lower than KRN 7000, whereas the TCR binding was severely affected. The stimulation of the NKT cells was certainly favoured by the presence of the hydroxyl in position 3′ but was shortened as the ternary complex was not very stable.

3.1.2. b) Modifications of the lipid chains. Truncation and/or functionalization of the lipid chains have shown very interesting results in terms of selective NKT cell response. The first analogue that significantly changed the response of NKT cells was called OCH (**2**) (Table 4). Its phytosphingosine chain contained only 5 carbons and the acyl chain 23 carbons, which gave a specific T_H2 activity[9,68c,72] but a weak stimulation on human *i*NKT cells.[73] At the same time, Goff *et al.* showed that the truncation of either the phytosphingosine (**47**) or the acyl (**48**, **49**) chains gives compounds with the same activity as OCH (Table 4).[74] From studies of KRN 7000 and OCH, Oki *et al.* have suggested that truncation of

Table 4 Truncation and/or functionalization of the lipid chains



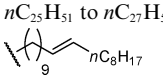
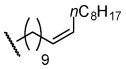
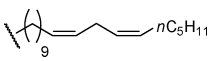
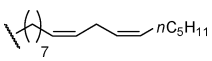
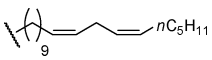
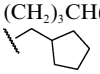
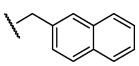
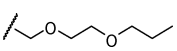
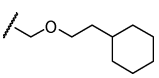
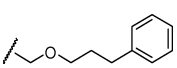
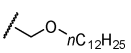
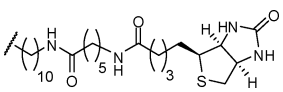
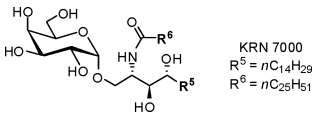
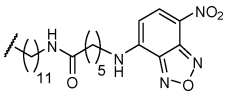
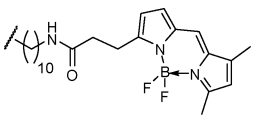
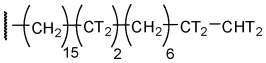
Analogues	R^5	R^6	Activity	Ref.
2 (OCH)	nC_5H_{11}	$nC_{23}H_{47}$	T_H2^{m+}, weak *i*NKT stimulation[h]	68c, 73
47	nC_2H_5	$nC_{23}H_{47}$	T_H2 (~↗)[m]	74
48	$nC_{14}H_{29}$	CH_3	T_H2 (↘↘)[m], T_H2 (↗)[h]	74
49	$nC_{14}H_{29}$	nC_7H_{15}	T_H2 (↗)[m,h]	74
50	nC_5H_{11}	$nC_{19}H_{39}$ to $nC_{24}H_{49}$	T_H2 (~→)[m]	75
51	nC_5H_{11}	$nC_{25}H_{51}$ to $nC_{27}H_{55}$	T_H1 (~→)[m]	75
52 (C20:1 *Trans*)	$nC_{14}H_{29}$		T_H2^{m+}	76
53 (20 : 1 *Cis*)	$nC_{14}H_{29}$		T_H2^{m+}	76
54 (C20:2 *Cis*)	$nC_{14}H_{29}$		T_H2^{m+}, potent response[h]	73, 76
55 (C18:2 *Cis*)	$nC_{14}H_{29}$		T_H2^{m+}	76
56[a]	nC_5H_{11}		T_H2 (→)[m]	73
57	$(CH_2)_3CH(CH_3)_2$	$nC_{21}H_{43}$	T_H1 (↘)[m]	77
58		$nC_{23}H_{47}$	T_H2 (→)[m]	75
59	CH_2Ph	$nC_{23}H_{47}$	T_H2 (→)[m]	75
60	$(CH_2)_2Ph$	$nC_{25}H_{51}$	T_H1 (↗↗)[m+,h]	62
61	$(CH_2)_4Ph$	$nC_{25}H_{51}$	T_H1 (↗)[m+,h]	62
62	$(CH_2)_3Ph$	$nC_{21}H_{43}$	T_H1 (↘)[m]	77
63	$nC_{14}H_{29}$	$nC_{21}H_{43}$	T_H1 (↘)[m]	77
64	$(CH_2)_3Ph$	$nC_{25}H_{51}$	T_H1 (→)[h]	79
65	$nC_{14}H_{29}$	$(CH_2)_5Ph$	T_H1 (↗)[m+,h]	62
66	$nC_{14}H_{29}$	$(CH_2)_7Ph$	T_H1 (↗↗)[m+,h]	62
67	$nC_{14}H_{29}$	$(CH_2)_{10}Ph$	T_H1 (↗↗)[m+,h]	62
68	$nC_{14}H_{29}$	$(CH_2)_7Ph$(*p*-F)	T_H1 (↗↗)[h]	78b
69	$(CH_2)_3Ph$	$(CH_2)_5Ph$	No *i*NKT cell activation	79
70	$(CH_2)_3Ph$	$(CH_2)_7Ph$	No *i*NKT cell activation	79
71	$(CH_2)_3Ph$	$(CH_2)_9Ph$	No *i*NKT cell activation	79
72	$nC_{14}H_{29}$		T_H1 (~↗)[h]	78a
73	$nC_{14}H_{29}$		T_H1 (~→)[h]	78a
74		$nC_{25}H_{51}$	*i*NKT cells activation (→)[m]	9b
75		$nC_{25}H_{51}$	*i*NKT cells activation (~↘)[m]	9b
76		$nC_{25}H_{51}$	*i*NKT cells activation (~↘)[m]	9b
77		$nC_{25}H_{51}$	*i*NKT cells activation (~↘)[m]	9b
***nn*78**	$nC_{14}H_{29}$		*i*NKT cells activation (↘)[m]	81a

Table 4 *(Contd.)*



Analogues	R^5	R^6	Activity	Ref.
79	$nC_{14}H_{29}$		*i*NKT cells activation (↗)[m]	81b
80	$nC_{14}H_{29}$		NC	81c
81	$nC_{14}H_{29}$		NC	82
82	$nC_{14}H_{29}$	$nC_{17}H_{35}$	NC	83

↘ Less potent than KRN 7000 or OCH according to T_H1 or T_H2 induced response; ↗ more potent than KRN 7000 or OCH according to T_H1 or T_H2 induced response; → similar activity compared to KRN 7000 or OCH according to T_H1 or T_H2 induced response; ~↗ or ~↘ slight increasing/decreasing; [m] *in vitro* tests performed on mice cells; [m+] *in vivo* tests performed on mice cells; [h] *in vitro* tests performed on human cells; **NC**: not communicated. [a] Sphinganine analogue (4′-deoxy).

one lipid chain leads to a less stable complex with CD1d and that IFN-γ release by NKT cells requires longer stimulation by CD1d/glycolipid complexes than IL-4 release.[46a] In 2007, Cerundolo and co-workers extensively studied how the length of the glycolipids bound to human CD1d molecules influenced the affinity of NKT cell TCR and the threshold of NKT cell activation.[46b] They showed that shortening either the acyl chain or the phytosphingosine one increased the rate of lipid dissociation from human CD1d molecules. Moreover, these types of analogues had to be introduced at a higher concentration than the KRN 7000 one to reach the threshold of NKT cell activation. Cerundolo's team hypothesized that the difference in TCR binding affinity between α-GalCer and studied OCH analogues ($R^5 = nC_8H_{17}$ and $nC_{11}H_{23}$) could be explained by a repositioning of this lipid chain in the human CD1d C' channel due to the shorter chain length. This movement might thus directly alter the position of the α-linked D-galactose or cause an amino acid shift orientation in the helices of the protein as a result of a collapse of the partially unfilled C' channel (Fig. 2). These major alterations have been observed on some residues located on the α1 helix and serving as TCR anchors and not on amino acids on the α2 helix as Cerundolo and co-workers suggested. Indeed, in our last dynamic simulations, we indicated that the lack of a lipid chain, in the C' hydrophobic channel of CD1d binding the OCH analogue, causes the deviation of Phe84 (interacting with CDR3α) that induces a poor structural alignment of the amino acids involved in the TCR recognition mediated by CDR2β (Glu83, Lys86, Arg89). A new orientation of the OCH sugar moiety has thus been observed that has a dramatic consequence on the H-bond network with CD1d and the possible interaction with the TCR (Fig. 9).[30]

The truncation of one lipid chain does not seem to be necessary and is not sufficient to inevitably observe a T_H2 response. Indeed, Annoura and co-workers have demonstrated that the production of IFN-γ noticeably increased in the case of analogues having a

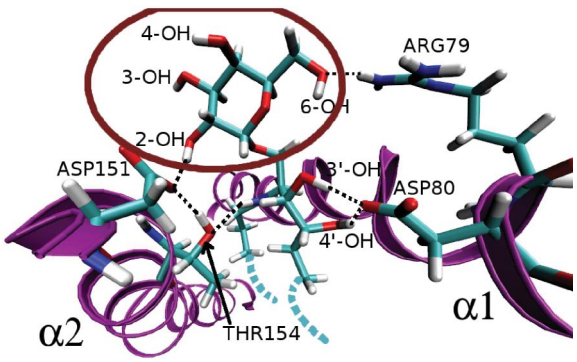


Fig. 9 New orientation of the OCH sugar head observed during a simulation of the binary complex.

shortened sphingosine chain and more than 24 carbons on the acyl moiety (**50**, **51**, Table 4).[75] The insertion of *Z*-unsaturations in the acyl chain without truncation of the phytosphingosine part (**52–55**), proposed by Porcelli's team, induced a better production of IL-4 in favour of a T_H2 response, compared to the case of OCH.[76] These results may be explained by the preformed curve of the acyl chain, due to the presence of the double bonds, which quickly stabilizes lipid binding by favouring the natural turning conformation into the CD1d cavity.[30,46b] However, when the sphingosine chain only contains 5 carbons, the corresponding analogue **56** offers no advantage over OCH.[73] From our previous theoretical study,[30] four positions in the acyl chain emerge on which the Z double bond may help to supply a rigid bend relatively close to that found in the natural compound. The insertion of double bonds in a glycolipid (**22**) also helps considerably to increase solubility, essential for biological tests, while keeping a similar activity compared to KRN 7000.[60a,46b,52]

Lately, the study of non-linear analogues was also investigated. Small moieties like isopropyl (**57**) in the γ position of the

4′-OH,[77] as well as a cyclopentyl (**58**)[75] or phenyl group (**59**)[75] in the α position of the 4′-OH on the phytosphingosine part did not improve activity (Table 4). Wong and co-workers have thus studied the insertion of both short and medium chains with terminal aromatic groups on both.[62,78] On the phytosphingosine part, the addition of a few methylene units (2 and 4) (**60**, **61**) as a spacer proved to be enough to induce a significant T_H1 response.[62] The binary complex was, indeed, well stabilized by a π-stacking interaction with Phe18 and Phe77, covering the entry of the hydrophobic pocket C', and the terminal phenyl on the glycolipids. This observation is even more obvious when the spacer is reduced to 3 methylene groups. In this case, stabilization is less efficient and the analogue (**62**) has a lower activity than KRN 7000.[77] Nevertheless the activity decrease can also be caused by truncation of 4 carbons on the acyl chain as observed with glycolipid **63**.[77] Indeed, the analogue **64** having 25 carbons on its acyl chain has shown a similar activity to KRN 7000.[79] Thus the insertion of an aromatic moiety on the phytosphingosine part seems to be very promising to increase the production of IFN-γ. This phenomenon is more noticeable when the modifications are done on the acyl chain. The elongation of the spacer chain length with 5 to 10 methylene groups (**65–67**) allowed a better interaction with the aromatic residues in the CD1d hydrophobic groove, and drastically enhanced the overall cytokine production.[62] More recently, the same team noticed a positive effect of the fluoro group in the *para* position on the terminal phenyl group (**68**) in the production of IFN-γ (Table 4).[78b] Aromatic analogues (**60**, **66**, **67** and **68**) have thus shown the most relevant T_H1 bias ever reported in the *O*-analogue series. Following this idea, Park *et al.* recently synthesized several analogues (**69–71**) having a terminal aromatic ring on their both chains, linked by 5 to 9 methylene groups on the acyl part and 3 methylene groups on the phytosphingosine moiety. Surprisingly, none of the compounds showed any NKT cell activation.[79] The insertion of heteroaromatic or naphthalene moieties was studied as well, but only the 2-thienylacetyl analogue (**72**) and the 2-naphthylacetyl derivative (**73**) have demonstrated an almost equal or better cytokine secretion than α-GalCer.[78a] Annoura and co-workers have also synthesized different non-linear moieties on the acyl chain, but no activities have been reported.[80]

Another interesting category of analogues was recently proposed by Panza and co-workers.[9b] They synthesized four new ether derivatives on the phytosphingosine part (**74–77**). The first biological assessments revealed a similar activation of *i*NKT cells compared to KRN 7000 for **74**. Apparently, the presence of two oxygen atoms in the chain does not have a major impact on the activity. Moreover, an aromatic moiety (**76**) did not give a better stimulation. Fluorescent moieties (**78–80**) have been also incorporated on the end of the acyl chain for studying metabolism and glycolipid traffic in animal cells.[81] Finally, tritium labelling on the acyl chain (**81**) has been performed to study *in vivo* α-GalCer pharmacokinetic properties.[82] We have also added in Table 4 another analogue (**82**) reported in the literature without any details about its activity.[83]

3.1.2. c) Amide side chain substitution and modification. This modification has been little studied in the literature and has nevertheless shown quite surprising biological results. Tashiro *et al.* have shown that the substitution of the acyl chain with a sulfonamide linkage allowed an IL-4 biased activity (**83** called RCAI-26, Fig. 10).[84] In 2007, Lee *et al.* found that the replacement of an amide function with an isosteric group such as a triazole increased the IL-4 *versus* IFN-γ bias of released cytokines.[85] The

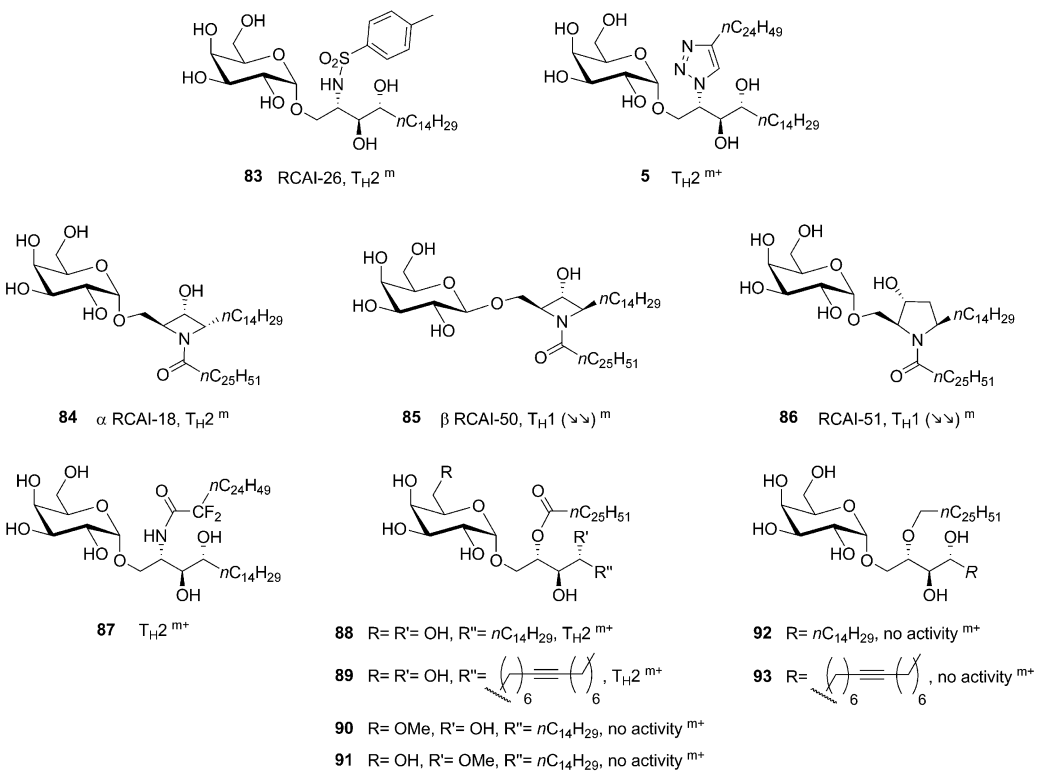


Fig. 10 Amide modified *O*-analogues.

stimulatory effect was influenced by the length of the attached chain. In particular, the long-chained triazole analogues (**5**) exhibited a stronger T_H2 cytokine response (*in vivo* and *in vitro* on mice cells). According to the docking model, the orientation of the glycolipid in the protein was quite similar to KRN 7000 and the triazole function maintained the H-bond network in place. However it is worth noting that the hydrogen bond between the 2-OH on the sugar and Asp151 was lost. As this hydroxyl group is free, it can participate in the recognition with the TCR (Fig. 3 and 5) and might be important for the induced T_H2 bias.

Another group in Mori's lab worked on conformationally restricted analogues by substituting the amide function on the ceramide moiety with azetidine or pyrrolidine rings.[86] The synthesis was based on a strategy which they had previously used in the syntheses of penaresidins A and B and penazetidine A.[87] Eight new analogues were synthesized but they all had lower activities than KRN 7000. However, among them, RCAI-18 (**84**), an azetidine derivative, has shown an interesting dose-dependent bioactivity with a T_H2 bias at low concentration, probably due to the decreased stability of the binary complex ligand/CD1d. It is worth mentioning that the analogue RCAI-50 (**85**) induces a slight IFN-γ production although the sugar is in a β configuration. Ring extension did not offer better bioactivity. Indeed, in RCAI-51 (**86**), the pyrrolidine derivative showed a decreased amount of cytokine induction compared to RCAI-18 and KRN 7000 (Fig. 10).

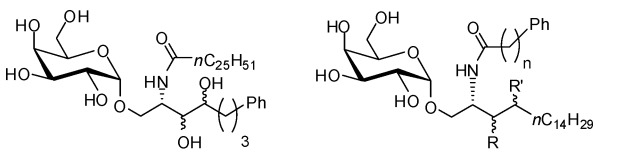
Recently, Linclau and co-workers have investigated the functional role of the interaction between the Thr154 on the α2-helix and the amide by altering the N–H hydrogen bond characteristics.[88] The fluorination of amides in the α-position increases N–H acidity,[89] and should consequently reinforce the H-bond network and, thus, offer a higher stable binary complex to preferentially induce a T_H1 cytokine polarisation. However, this new fluorinated analogue **87** unexpectedly induced a T_H2 bias. The authors suggested that the hydrogen bond NH/Thr154, if indeed stronger, plays no major role in stabilising the GalCer in the CD1d protein. Another hypothesis could be that the fluorine introduction, which can involve a shallow torsion of the acyl chain due to an antiperiplanar conformation of the C–F bond to the C═O bond,[90] might affect the H-bond network OTAN.[30] This in turn could negatively impact the polar group stabilisation, which is consistent with a lower affinity with the TCR and a T_H2 bias.[85]

In 2010, the replacement of the amide function by an ester or ether group was envisaged by Shiozaki *et al.*[91] They prepared 4 ester analogues (**88–91**) and 2 ether derivatives (**92**, **93**) in 10–12 steps from L-Ribose and a thio-α-galactoside derivative with poor overall yields (0.2–1.3%) (Fig. 10). During the syntheses of the ester analogues, they observed an interesting migration of the long lipid ester chain from the 2′ position to the 4′ position in acidic conditions. Biological tests performed showed that the ether derivatives **92**, **93** and the ester analogue possessing a methoxy group in 4′ position (**91**) are completely inactive on murine cells *in vivo*, whereas the ester analogues **88–90** are active with a weak but promising T_H2 immune response. Overall, it seems that the lack of the carbonyl group causes a difference in the geometry and orientation of the compound inside CD1d, and has a direct impact on the activity. Moreover, replacement of the nitrogen with an oxygen certainly induces a slight destabilisation of the OTAN network, and probably alters the position of other amino acids engaged in the recognition with the TCR (as is the case for the OCH compound). The migrated ester derivative was unfortunately not tested.

The majority of amide modified compounds have shown a relevant T_H2 induction and are of real interest for the investigation of autoimmune disease treatments. Nevertheless, the modification of this moiety is in the early stages and should still be investigated. The use of docking models could help in the understanding and design of new promising analogues.

3.1.3 Miscellaneous modifications.

3.1.3. a) Modifications combining stereochemistry and aromatic moieties. Following their former studies cited above, Park *et al.* recently prepared a series of analogues in which various combinations of the aromatic residue in both chains and the backbone stereochemistry were made.[79] The impact of these modifications on the activity was essentially driven by the presence of the phenyl groups on the chains as the chosen orientation of the amide and hydroxyl groups ((2′*S*, 3′*R*, 4′*S*), (2′*S*, 3′*S*, 4′*S*), (2′*S*, 3′*R*, 4′*R*)) do not cause any change in the activity.[65] Unsurprisingly, these new compounds did not show any NKT cell activation as previously observed for the analogues **69–71**. A similar activation of NKT cells compared to KRN 7000 was again obtained for compound **94** in which the usual linear acyl chain (25 carbons) was reinstalled. However, the production of relevant cytokines was lower than in the case of KRN 7000. The same observations were made when the phenyl group was then placed on the acyl chain while the normal sphingosine chain was used (**95**, **96**) or even when the 4′-OH group was missing (**97**, **98**) (Fig. 11). Finally, this study showed that the lack of hydroxyl groups can be remarkably offset by the presence of a terminal aromatic ring spaced 9 methylene groups from the amide, this analogue inducing a noticeable T_H2 bias response (**99**). Thus, careful insertion of phenyl groups in the lipid chains could be another versatile strategy to preferentially obtain T_H2 activity.

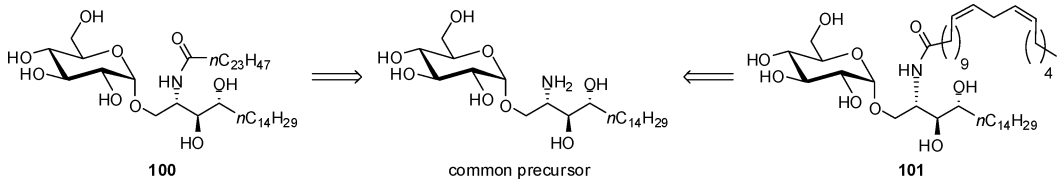


94 (3'S, 4'S), T_H1 (↘↘) [h]

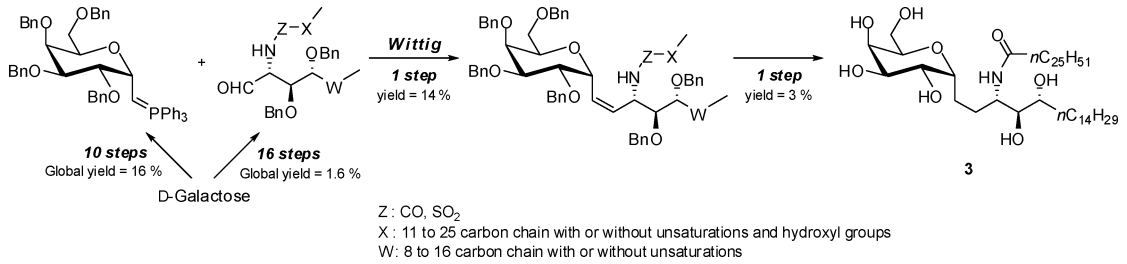
95 n= 5, R= R'= OH (3'S, 4'S), T_H1 (↘↘) [h]
96 n= 9, R= R'= OH (3'S, 4'S), T_H1 (↘↘) [h]
97 n= 5, R= OH (3'S), R'= H, T_H1 (↘↘) [h]
98 n= 9, R= OH (3'R), R'= H, T_H1 (↘↘) [h]
99 n= 9, R= R'= H, T_H2 [h]

Fig. 11 Modified analogues combining stereochemistry and aromatic moieties.

3.1.3. b) Combination of structural modifications on the sugar and the acyl chain. Lately, Jervis *et al.* have imagined a short and efficient strategy using an amine as a common precursor to prepare two new analogue compounds (**100** and **101**) possessing a D-glucose moiety and a saturated or unsaturated acyl chain respectively (Scheme 2).[92] Initial biological studies have shown that these α-glucosylceramides provided a similar stimulation of human *i*NKT cell response compared to KRN 7000. These results are similar with previous observations concerning the influence of the type of the sugar on mouse T cell activation.[39a,53] Here the D-glucose part seems to be quite suitable to be recognised by



Scheme 2 New saturated and unsaturated α-glucosylceramides.



Scheme 3 Synthesis of α-*C*-KRN by Kirin.

human CD1d. Moreover, it would be interesting to know if the unsaturated ligand **101** can keep or improve the T_H2-bias tendency observed in the case of its D-galactoside counterpart **54** (see Table 4) to seriously consider it as a strong contender for further clinical developments as an immunomodulator.

3.2. *C*-glycosides analogues

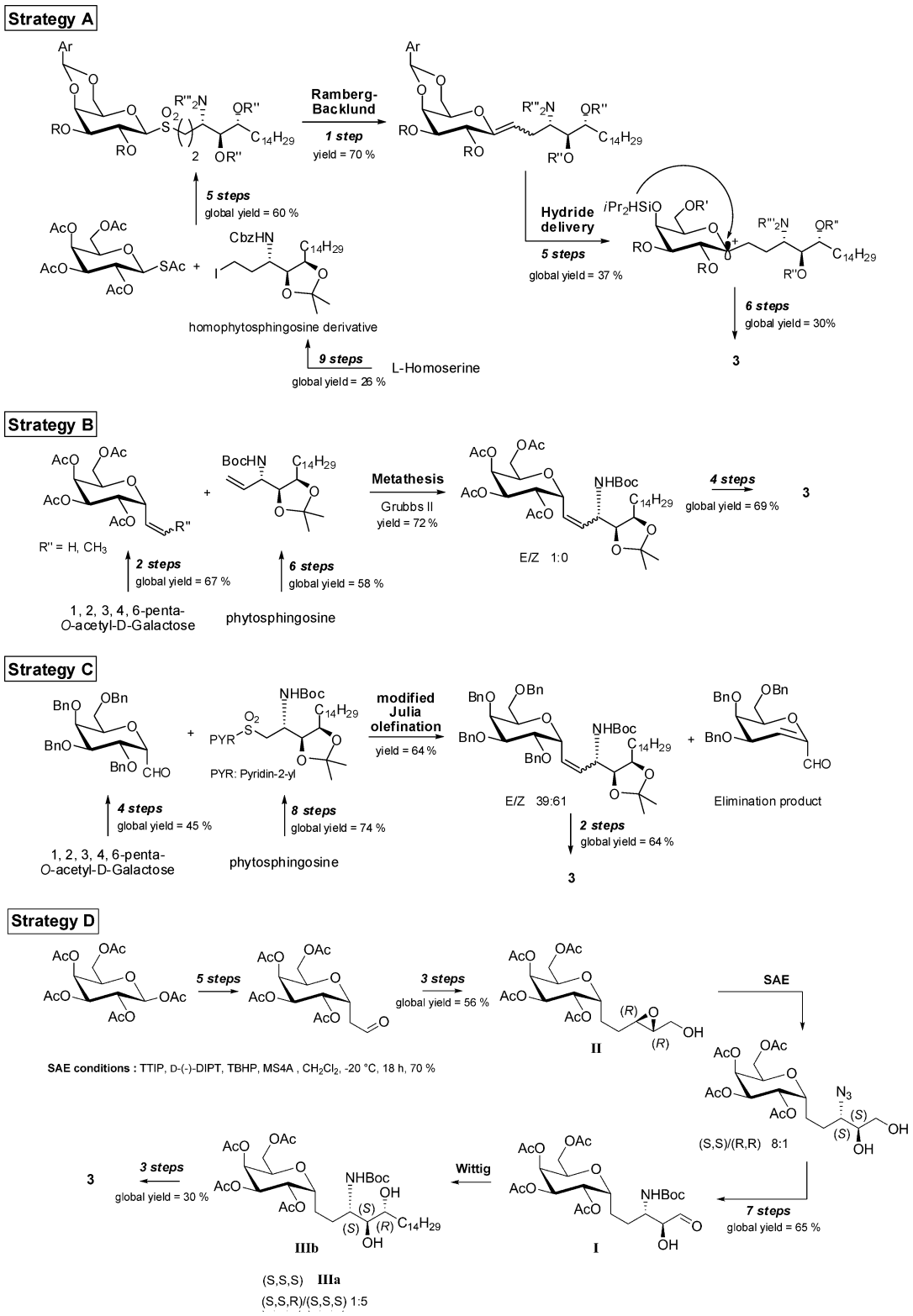
Another obvious modification has been to change the nature of the glycoside bond with a methylene group in order to limit the *in vivo* degradation of the glycolipid by glycosidases. In 2002, the first biological tests carried out by Kotobuki Pharmaceutical Co. with α-*C*-KRN (**3**) showed a stronger T_H1 response compared to KRN 7000 against melanoma B16.[93] To the best of our knowledge, α-*C*-KRN is the only *C*-glycoside known to be more active than its parent *O*-glycoside. Recent detailed biological tests have shown that it is indeed approximately one hundred times more potent than KRN 7000 against mouse lung metastasis at a microgram scale and a thousand times more effective against mouse malaria assay at a nanogram scale.[94] However, Tsuji and co-workers surprisingly showed that the human CD1d dimer loaded with α-*C*-KRN did not strongly interact with human *i*NKT cells. Consequently α-*C*-KRN was unable to induce a significant level of cytokine production by these cells.[31] According to the authors, the solubility of the glycolipid is not responsible for the observed lack of activity, as the α-*C*-KRN can be loaded in both human and murine CD1d molecules despite their structural differences. Apparently, the differences between human and murine *i*NKT cells seem to be more critical to induce activity than those between human and murine CD1d molecules.[95]

3.2.1. Syntheses of *C*-KRN 7000 and modelling studies.

3.2.1. a) Different synthetic approaches. Currently, six syntheses of *C*-KRN (**3**) have been reported in the literature. Some were original, short and efficient, others were longer and did not allow easy access to a large library of analogues. The convergent strategy proposed by Kotobuki Pharmaceutical Co. in 2002 consists of a Wittig reaction as the key step to unify the α-D-galactose moiety with the entirely functionalized ceramide part (Scheme 3).[93] Unfortunately, this way was long (18 steps), poorly reproducible,[96] and led to α-*C*-KRN with a very low yield and with limited structural modifications being possible.

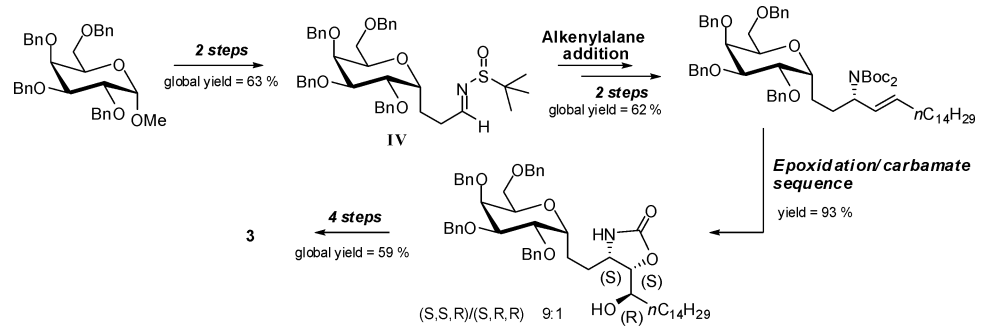
During the last five years, Franck's team has published four original syntheses of *C*-KRN, which have also given access to new α-*C*-KRN analogues (see 3.2.2. a)). As key steps, the first method used the Ramberg-Bäcklund reaction to generate the *C*-glycoside bond, followed later by a hydride transfer to achieve the α configuration (Scheme 4A).[94] However this strategy was long (26 steps), with a very low yield and the functionalization of the lipid chains occurred earlier in the synthesis. The second synthesis presented a more convergent, short (11 steps) and efficient (30% yield) strategy featuring cross-metathesis as a key step (Scheme 4B). An alternative route was also developed involving a modified Julia olefination (Scheme 4C) in order to increase the ratio of the *cis* isomer for further studies.[94c,97b] This method inconveniently gave a greater fraction of elimination product. Moreover the use of expensive phytosphingosine as starting material in both syntheses B and C might limited the scale-up procedure.[97] The last synthesis reported is based on the Sharpless asymmetric epoxidation (SAE) in order to obtain a pre-formed *C*-glycoside material (**I**) and access to various phytosphingosine chains.[98] The epoxide opening key step (**II**) with sodium azide was not highly regioselective but finally led to the expected key aldehyde (**I**) in 16 steps (Scheme 4D). Unfortunately, the Wittig reaction used to insert the lipid chain was poorly stereoselective in favour of the α-*C*-KRN precursor (**IIIb**). This fourth way proposed by Franck and co-workers is thus poorly regio- and stereoselective, long (20 linear steps) and leads to α-*C*-KRN (**3**) in a low overall yield (3.3%).

Another linear synthesis of **3** was proposed by Wipf and Pierce in 2006.[99] Key steps involved an alkenylalane addition to an *N*-tert-butanesulfinyl imine **IV** followed by an epoxidation/carbamate ring opening sequence to install the diol (Scheme 5). This strategy was shorter (only 10 steps), efficient (21% global yield) and versatile, allowing derivatization of the acyl chain, despite a low diastereoselectivity during the epoxidation and the use of an expensive protected sugar.

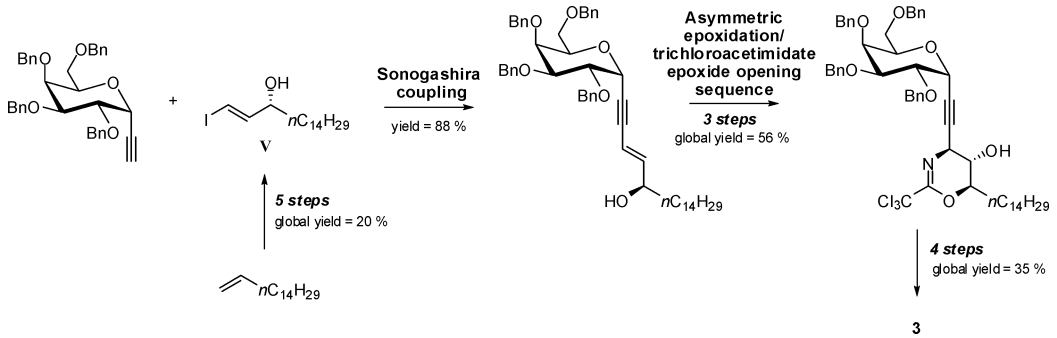


Scheme 4 Proposed syntheses by Franck and co-workers.

Finally, in 2010, Bittman and co-workers published a new convergent synthesis of α-*C*-GalCer in 15 steps in just above 1% overall yield.[100] By using a Sonogashira coupling as the first key step, the protected D-galactose derivative was successfully joined in the desired α configuration to the precursor **V** of the ceramide part. The insertion of the hydroxyl in position 3′ and the amine were achieved with an asymmetric epoxidation followed by a trichloroacetimidate-mediated epoxide opening sequence



Scheme 5 Wipf and Pierce's strategy.



Scheme 6 Bittman's strategy.

(Scheme 6). This strategy presents the advantage of using simple starting materials and allows the preparation of some analogues with variations in the glycoside linker area.

3.2.1. b) Toward modelling studies. As suggested by Franck and co-workers, the activity of α-*C*-GalCer observed against mouse *i*NKT cells, but not against human *i*NKT cells suggests that the O/CH_2 group switch in the linker region causes one of two possible alterations: 1) distortion of the D-galactose, so that the sugar head group of α-*C*-GalCer is accessible to the semi-invariant TCR of murine *i*NKT cells, but does not fit as well with that of human *i*NKT cells; or 2) the CH_2 induces a change in conformation of the CD1d-binding surface, so it is less recognizable to the TCR of *i*NKT cells.[31] Surprisingly, preliminary MD simulations performed on the binary complex involving α-*C*-GalCer in our laboratory did not reveal any modification in the orientation of the ligand in the CD1d compared to KRN 7000. Further investigations are currently in progress on the ternary complex to elucidate the binding features with the TCR as the strong T_H1 bioactivity of α-*C*-GalCer seems to be unrelated to a particular recognition with CD1d.[101]

3.2.2. Modification of the ceramide moiety.

3.2.2. a) Modification of the backbone structure. Kirin Pharma has described some modifications in their patent. However, no biological results have been reported for these analogues (Fig. 12).[93]

The **102**-type analogues proposed by Kirin were fully developed by Franck and co-workers during their second synthesis of the α-*C*-KRN.[94c,97] These *C*-analogues, with an unsaturated double bond as a spacer between the D-galactose moiety and the ceramide, were tested, and the *E* analogue (**102**) showed a better T_H1 response than KRN 7000 but lower than *C*-KRN, whereas the *Z* material

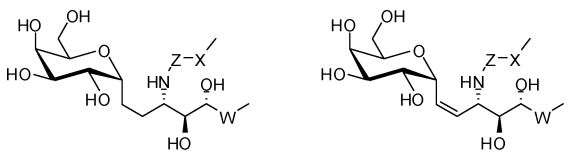
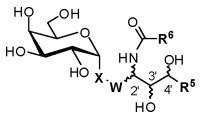


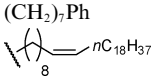
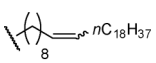
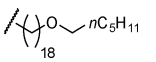
Fig. 12 Kirin *C*-type analogues.

(**103**) was inert. A loss of the activity was also observed when the unsaturated spacer (W position in Table 5) was increased by only one carbon (analogues **104**, **105**). Recently, a new series of **102**-type analogues (**107–111**) possessing a variety of fatty acid chains were synthesized and tested on mice and human NKT cells (Table 5). Their activity was compared to that observed for a α-*C*-Galcer analogue having a prolonged acyl chain ($nC_{29}H_{59}$), called CRONY (**106**).[31] Consistent with other bioassays on **3**, CRONY was found to be a very weak ligand for human *i*NKT cells but induced the maturation of murine DCs *via* activation of *i*NKT cells *in vivo*.[31] On the contrary, the new analogues **108** with an aromatic ring at the end of the shorter alkyl chain, and analogues **109** and **110**, with the addition of an olefin linkage in the alkyl chain, induced a higher level of T_H1 cytokine production by human *i*NKT cells compared with the standard **107**. Compound **111**, an analogue with an ether linkage failed to enhance any significant production of T_H1 cytokines. Most interestingly, although both **108** and **110** possessed strong biological activity against human *i*NKT cells, **110** also exhibited a potent *in vitro* stimulatory activity against murine NKT cells, while **108** failed to induce strong T_H1 cytokine production by murine NKT cells either *in vivo* or *in vitro*.

Table 5 Activity of α-*C*-GalCer and its analogues



KRN 7000 (2′S, 3′S, 4′R)
X = O, W = CH_2, $R^5 = nC_{14}H_{29}$, $R^6 = nC_{25}H_{51}$

Analogues	X	W	Double bond	R^5	R^6	Configurations 2′, 3′, 4′			Activity	Ref.
3 (*C*-KRN)	CH_2	CH_2	—	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*R*	T_H1 (↗↗) $^{m+}$, Weak activity^{h}	31, 93, 94,
102	CH	=CH-	E	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*R*	T_H1 (↗)$^{m+}$	31, 94c, 97
103	CH	=CH-	Z	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*R*	No activity	31, 94c, 97
104	CH_2	CH=CH-	E	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*R*		31, 94c, 97
105	CH_2	CH=CH-	Z	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*R*		31, 94c, 97
106 (CRONY)	CH_2	CH_2	—	$nC_{14}H_{29}$	$nC_{29}H_{59}$	*S*	*S*	*R*	*i*NKT activation $^{m,m+}$, No activity^{h}	31
107	CH	=CH-	E	$nC_{14}H_{29}$	$nC_{29}H_{59}$	*S*	*S*	*R*	$T_H1^{m,m+,h}$	31, 95
108	CH	=CH-	E	$nC_{14}H_{29}$	$(CH_2)_7Ph$	*S*	*S*	*R*	No activity$^{m,m+}$, T_H1^{h}	31, 95
109	CH	=CH-	E	$nC_{14}H_{29}$		*S*	*S*	*R*	$T_H1^{m,m+,h}$	31
110	CH	=CH-	E	$nC_{14}H_{29}$	 Z/E 20:80	*S*	*S*	*R*	$T_H1^{m,m+,h}$	31
111	CH	=CH-	E	$nC_{14}H_{29}$		*S*	*S*	*R*	No activity^{h}	31
112			—	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*R*	NC	100
113	CH_2	CH_2	—	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*S*	T_H1 (↘)^{h}	98
114	CH_2	CH_2	—	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*R*	*S*	*R*	T_H1 (↘)^{h}	98
115	CH_2	CH_2	—	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*R*	*S*	*S*	T_H1 (↘)^{h}	98
116	CH_2	CH_2	—	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*R*	*S*	NC	99
117	CH_2	—	—	$nC_{14}H_{29}$	$nC_{25}H_{51}$	*S*	*S*	*R*	T_H1 (↗)^{h}	102
118	CH_2	CH_2	—	nC_5H_{11}	$nC_{23}H_{47}$	*S*	*S*	*R*	No activity^{m}, T_H2^{m+}	96

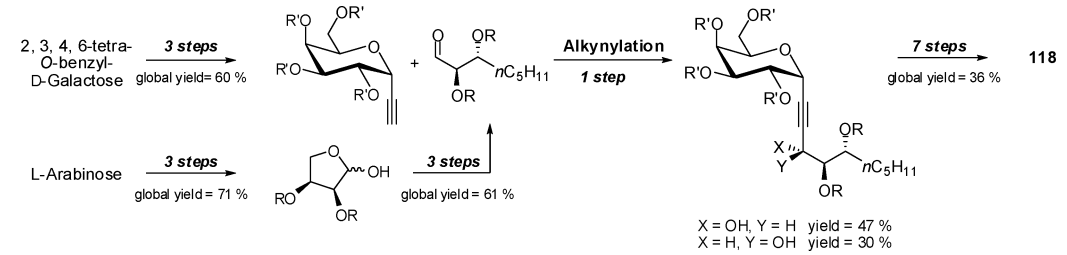
↘ less potent than KRN 7000 or OCH according to T_H1 or T_H2 induced response; ↗ more potent than KRN 7000 or OCH according to induced T_H1 or T_H2 response; → similar activity compared to KRN 7000 or OCH according to T_H1 or T_H2 induced response; ~↗ or ~↘ slight increasing/decreasing; ^{m} *in vitro* tests performed on mice cells; $^{m+}$ *in vivo* tests performed on mice cells; ^{h} *in vitro* tests performed on human cells; **NC**: not communicated.

This observation raises the problem of the structural differences between the mouse and human CD1d molecules and TCR cells, involving different ligand recognition.[95] The *E*-olefin linker has the advantage of having less steric repulsion energy than an O or CH_2 spacer.[31] The presence of the *E*-olefin spacer instead of the CH_2 linkage modifies the angle of the adjacent bond and should have a greater influence on the orientation and stabilization of the ligand in the CD1d. The angle fixed at 180° by the *E* conformation matches precisely with the dihedral angle (about 170°) observed between the D-galactose and the ceramide in the X-ray structure. Thus, even *E* analogues fit well into the CD1d groove, and the structural difference of CD1d molecules appears to be less critical than the structural difference of *i*TCR cells in shaping the strength of the biological activity of these kinds of compounds.[95] In the same effort to keep a rigid structure in the linker area, Bittman and co-workers have recently synthesised a α-*C*-acetylene analogue **112**, not yet tested.[100]

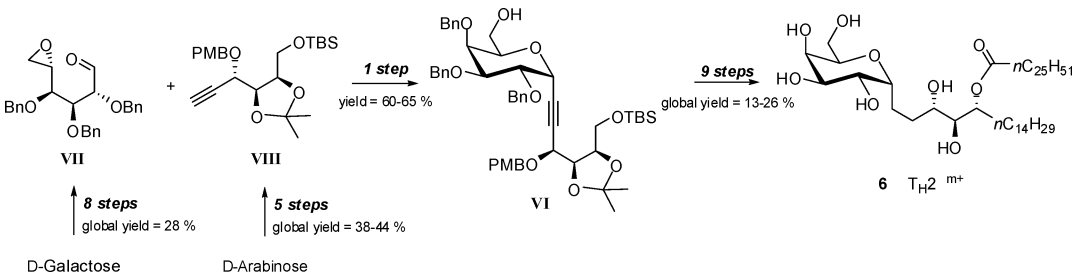
Several diastereoisomers were also obtained by Franck's team during their studies on the use of SAE reactions to prepare *C*-glycosyl derivatives (see Scheme 4D).[98] The diastereoisomers (2′*S*, 3′*S*, 4′*S*) (**113**), (2′*R*, 3′*S*, 4′*R*) (**114**) and (2′*R*, 3′*S*, 4′*S*) (**115**) were separated and tested (Table 5). These unnatural compounds were less active than KRN 7000 and the results close to those observed for the *O*-GalCer stereoisomers (see Table 3). Wipf *et al.* have also isolated the (2′*S*, 3′*R*, 4′*S*) isomer (**116**) which was obtained due to a low diastereoselectivity in their synthesis (see Scheme 5). However, no biological data were reported for this isomer.[99]

It is known that increasing the length of the spacer between the sugar and the ceramide by only one carbon induces a loss of activity. Bittman and co-workers synthesized a new truncated non isosteric α-*C*-Galcer analogue (**117**) which, like *C*-KRN, is not enzymatically labile at the glycoside linkage, and the anomeric carbon is directly bonded to the phytoceramide backbone.[102] *In vitro*, **117** was a less potent agonist for NKT cells than its parent, but it induced cytokine production with the highest IFN-γ/IL-4 and IFN-γ/IL-13 ratios typical of a T_H1 immune response (Table 5).

The expected *C*-glycoside OCH analogue (**118**) was prepared by Annoura's team in 14 steps in 7% global yield.[96] The strategy



Scheme 7 Annoura's synthetic strategy.



Scheme 8 New *C*-glycoside ester analogue **6**.

involved an alkynylide addition to a phytosphingosine type aldehyde, but the coupling was not stereoselective and 15% of starting material was recovered (Scheme 7). Moreover derivatization of the phytosphingosine chain occurred early in the synthesis, limiting the access to a small library of analogues. The *C*-glycoside OCH (**118**) did not show IL-4 and IFN-γ production *in vitro* in splenocytes but increased serum levels of IL-4 in C57BL/6 mice *in vivo* as its parent (**2**) (Table 5).

3.2.2. b) Amide side chain substitution in the C*-analogue series.* Our goal in the research of potentially bioactive analogues of KRN 7000 was to develop an easy synthetic access to the α-*C*-KRN 7000 skeleton by using a strategy developed in our laboratory based on a *nucleophilic* addition followed by an *epoxide opening* (the NEO strategy).[103] The functionalized *C*-galactoside framework **VI** chosen as a strategic intermediate could thus be obtained in a one pot procedure in good yield (60–65%) from an epoxyaldehyde **VII** and a functionalized alkyne **VIII**, both obtained from inexpensive sugars.[104] In order to generate a large library of analogues, the insertion of lipid side chains was planned at an advanced stage in the synthesis (Scheme 8).

Following the promising amide modified series, we also envisaged to prepare a new analogue in which the amide function was replaced with an ester. Moreover, this compound was also an interesting precursor of thionoester derivatives.[105] After functionalization of the key intermediate **VI**, the deprotection of hydroxyles 3′-OH and 4′-OH under strongly acidic conditions led us to the rearranged compound **6** in which the long lipid ester chain has migrated to the alcohol in the beta position as previously described by Shiozaki *et al.*[91]

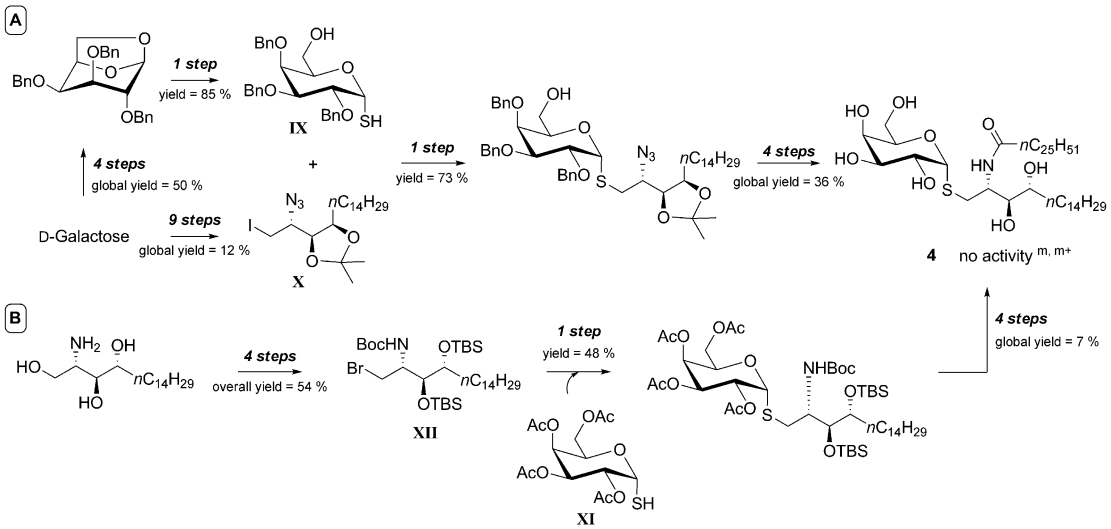
Preliminary *ex vivo* and *in vivo* biological tests performed on this unusual ester analogue, showed that **6** can also stimulate NKT cells and induce an encouraging T_H2 biased response, even if the *in vivo* cytokine production remained weaker than in the case of KRN 7000. Following our previous theoretical work,[30] molecular modelling studies are currently under progress to evaluate the impact this particular ester could have on the stabilization and the orientation of the CD1d/glycolipid/TCR complex.

3.3. Other types of glycoside linkers

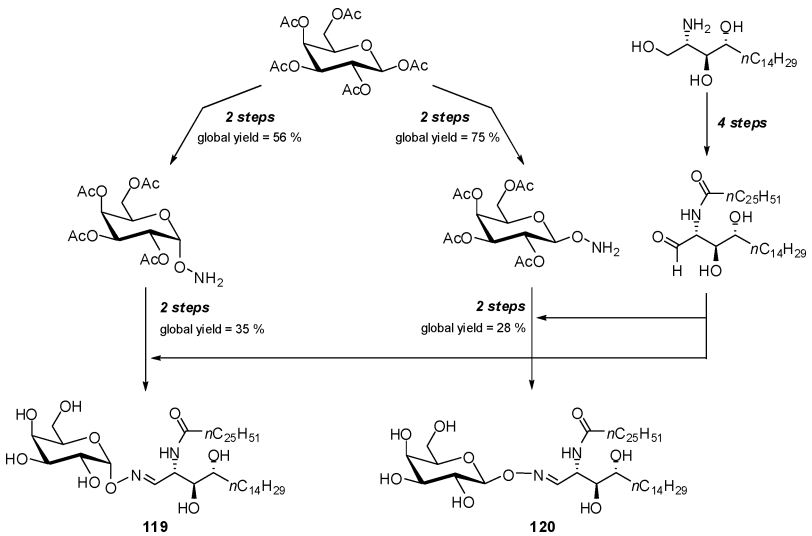
3.3.1. *S*-glycoside analogue. Recently, two syntheses of a new analogue in which the anomeric oxygen atom has been replaced with a sulfur to obtain the corresponding thioglycoside have been reported. Zhu and co-workers prepared the new isostere compound **4** in 14 steps starting from D-galactose in 3% overall yield (Scheme 9A).[106] Based on the model of the KRN synthetic route B (see Scheme 1), their strategy involved the α-galactosyl thiol **IX** and an electrophilic lipid building block **X**, also obtained from D-galactose. The single α anomer **IX** resulted from a ring opening procedure, and the coupling was then performed under mild phase transfer conditions in a good yield without preliminary protection of the 6-OH group.

The second synthesis reported by M. L. Blauvelt *et al.* involved a substitution approach between the thiol sugar **XI** and a brominated derivative **XII** of ribo-phytosphingosine.[107] The α and β anomers were separated after the coupling reaction and final deprotection led to the expected product **4** obtained in 9 steps and in 2% overall yield (Scheme 9B).

Chemically and catabolically stable, *S*-linked glycoconjugates are tolerated by most biological systems.[108] Thus, the new *S*-glycolipid **4** might remain active *in vivo* for a longer period of time. Unfortunately, biological tests have shown a total inactivation of the *i*NKT cells both *in vitro* or *in vivo*.[107] Their modelling studies showed the loss of some hydrogen bonds, especially those involving Thr154 and the nitrogen. Furthermore these results can be simply accounted for by the increased length of the glycoside bond. Indeed, the S–C bond (182 pm) is longer than the O–C one (143 pm). Two S–C bonds might induce a noticeable elongation of the spacer to cause a total loss of the activity as previously observed for compounds **104** and **105**.[97a]



Scheme 9 Syntheses of the new thiogalactosyl analogue **4**.



Scheme 10 Synthesis of new oxime derivatives **119** and **120**.

3.3.2. Oxime analogues. Chen *et al.* have recently developed new α and β analogues (**119** and **120**) possessing an oxime bond between the sugar and the ceramide.[109] The oxime linkage is also stable under physiological conditions, so these compounds are interesting because of potentially low hydrolysis rates in biological systems. However, it is worth noting that the presence of the nitrogen increasing the linkage by one unit might be detrimental for the bioactivity. Indeed, as previously seen with compounds **104** and **105**, this elongation induced a total loss of the activity.[97a]

With a convergent strategy, both α and β diastereoisomers have been prepared in 4 steps from a common starting material, the peracetylated D-galactose, in 19% and 21% yield respectively (Scheme 10). The disadvantage of this synthesis is the use of an expensive commercial phytosphingosine, thus limiting possible structural modifications of the lipid chains and the low yield of the coupling reaction. As the authors suggested, this strategy could nevertheless be efficient to couple various carbohydrates and ceramide lipids, provided that the bioactivity of such oxime derivatives is sustainable.

3.4. β-glycoside analogues

Obviously, the configuration of the glycoside bond has also been studied (Fig. 13). Koezuka and co-workers have thus observed that the β anomer (**121**) was less potent than KRN 7000.[24a,69] The same observation was made during the study of fluorescent analogues of KRN 7000 (**122**), a few years later.[81a] Moreover, the β anomer is not as active as sulfatides (**123**) or β-glycolipids found in myelin, and presented by all human CD1 family members to specific T cells.[110] The structural difference between all CD1 clusters[27] and the modification of the ligand orientation in the protein caused by the β conformation should be related to this random activity.[37] Nevertheless, several teams have undertaken the syntheses of different β-analogues to improve this weak activity. For example, 3-*O*-sulfo-β-KRN 7000 (**124**), built on the model of its α anomer having a similar activity to KRN 7000, has thus been synthesized but has unfortunately shown no activity for NKT cells.[57] Moreover, a truncated β-acylamide analogue containing a sphingosine type chain (**125**), synthesized in 1998, was less active

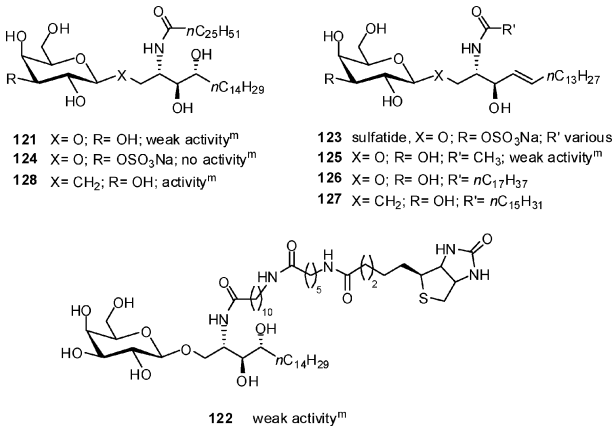


Fig. 13 Known β analogues.

than the α anomer.[111] Recently, the same type of compound with a longer acyl chain (**126**) was prepared, but no information about its activity was reported.[112] Finally, β-*C*-KRN analogues were also synthesized. The first methylene isostere (**127**) was obtained from sugar phosphorane in 1999 by Dondoni and co-workers.[113] And the last one (**128**) was prepared by Chaulagain *et al.* according to a ring-closing metathesis strategy.[114] For the moment, only *in vitro* assays have been performed on this analogue and have surprisingly shown a selective toxicity against solid tumor cells and leukemia cells without cytotoxicity against normal murine cells. These results are promising for the development of new potent antitumoral β analogues.

3.5. Other analogues: Carbaglycosides, inositol-, myoinositol-, serine-type derivatives and non-glycoside analogues

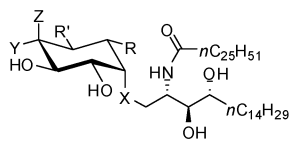
This section describes the various other KRN 7000-based analogues synthesized in the literature. Following works initiated by Chung and co-workers in 2006,[115] Mori's team achieved the synthesis of a carbocyclic analogue RCAI-56 (**7**) in order to evaluate the impact of the intracyclic oxygen in the sugar and to replace the glycoside linkage with a stronger ether bond (Fig. 14).[116] A docking model showed that the orientation of the ligand inside the murine CD1d was not disturbed and that **7** induced good T_H1 biased *in vivo* cytokine production by remarkably increasing the production of IFN-γ with a concomitant decrease in IL-4 release. The same biological profile has been observed with another carbosugar derivative **129** (RCAI-101).[117]

Based on the same idea, Cerundolo *et al.* have synthesized myoinositol derivatives (**130**, **131**), but they proved to be slightly less potent than KRN 7000 and seemed to have a lower affinity for the TCR (Fig. 14).[118] Mori and co-workers also observed a plunge in the bioactivity of their inositol analogues **132** (RCAI-37) and **133** (RCAI-102).[117]

New modified cyclitols **134–136** (RCAI-59, -60, -92), recently developed by Mori's team, have shown a remarkably stronger T_H1 activity than KRN 7000.[117] As the 6′-hydroxy group of the D-galactose part of KRN 7000 does not interact with CD1d or TCR, the modifications performed at this position were well tolerated as also observed for the sugar derivatives **17–22** and **24–27**. However, the aminocyclitol ceramide **137** (HS44) synthesized by Harrak *et al.* exhibited only a weak stimulatory activity against *i*NKT cells.[119]

A series of serine-type analogues (**138–140**) was synthesized by a Japanese team. The compound CCL-34 (**138**) was the only one of the series to show promising results notably in macrophage activation.[8d,120] The backbone of this new analogue is similar to the diacylglycerol structure of BbGL-II, another potent glycolipid which stimulates mice and human NKT cells.[10,12b,121]

Finally, non-glycoside analogues were also studied.[122] Since they lack the glycoside bond found in α-GalCer, they represent hydrolytically more stable compounds. Moreover, according to molecular modelling studies, they are able to keep different numbers of stabilizing H-bonds with Phe29, Ser30, and Gly96 residues in the TCR. The Threitolceramide (**141** ThrCer) showed the best



Carbasugar analogues (X= O)

7 RCAI-56; Y= R= H; Z= OH; R'= CH_2OH; T_H1 (↗) [m+, h]

129 RCAI-101; Y= R= H; Z= OH; R'= CH_2OMe; T_H1 [m+]

Myoinositol and inositol derivatives (X= O)

130 Y= H; Z= R= OH; R'= CH_2OH; T_H1 (~↘) [h]

131 Y= H; Z= OH; R= OSO_3Na; R'= CH_2OH; T_H1 (~↘) [h]

132 RCAI-37; Y= R= R'= OH; Z= H; almost inactive [m+]

133 RCAI-102; Y= H; Z= R= R'= OH; almost inactive [m+]

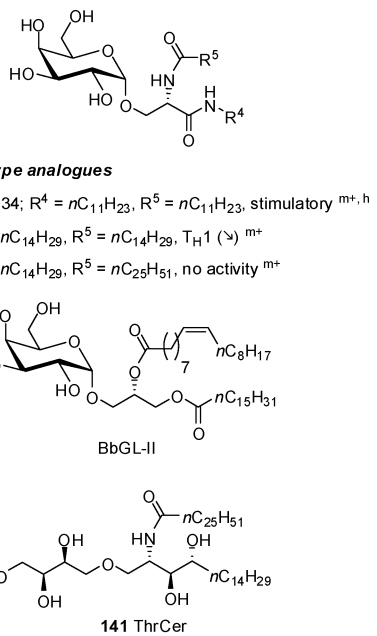
Modified cyclitol analogues

134 RCAI-59; X=O; Y= R= H; Z= R'= OH; T_H1 (↗) [m+, h]

135 RCAI-60; X= O; Y= R= H; Z= OH; R'= Me; T_H1 [m+]

136 RCAI-92; X=O; Y= R= H; Z= OH; R'= OMe; T_H1 (→) [m+]

137 HS44; X= NH; Y= R'= OH; Z= R= H; weak stimulatory [m+]



Serine-type analogues

138 CCL-34; $R^4 = nC_{11}H_{23}$, $R^5 = nC_{11}H_{23}$, stimulatory [m+, h]

139 $R^4 = nC_{14}H_{29}$, $R^5 = nC_{14}H_{29}$, T_H1 (↘) [m+]

140 $R^4 = nC_{14}H_{29}$, $R^5 = nC_{25}H_{51}$, no activity [m+]

141 ThrCer

Fig. 14 Other KRN 7000-based analogues and non-glycoside compound.

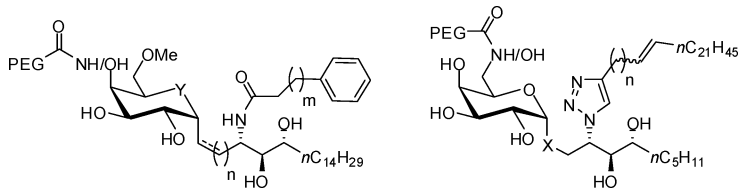


Fig. 15 Hybrid analogues.

selective expansion and activation of human and mouse *i*NKT cells compared to glycerol- and arabinitol-ceramide (Fig. 14).

4. Toward a selective T_H1/T_H2 immune balance? - Conclusions and perspectives

As can be seen with the numerous literature examples dealing with glycolipid analogues, this class of compounds continues to greatly arouse the interest of scientists. Among the substantial library, some analogues (OCH **2**, *C*-KRN **3**, triazole derivative **5**, RCAI-56 **7**, and compounds **57**, **64**, **87**, **95**) have shown promising results towards a selective immune balance, even if most of them preferentially induced a T_H1 response. In the last fifteen years, great advances have been made to build a good selective analogue and to understand the mechanism of the *i*NKT activation. SAR studies have determined a list of structural elements required for activity. Crystallographic studies have elucidated the conformation of the ternary complex formed by the CD1d, the glycolipid and the human *i*TCR. Breakthroughs in molecular modelling studies have given a useful tool to visualize and elaborate the design of envisaged analogues. The development of human biological assays is the final element necessary to reveal the most potent candidates for future therapy.

Looking at the structure of these analogues, we can postulate that, on one hand, the T_H1 immune response is certainly favoured by a stable glycoside bond, a rigid conformation of the spacer between the D-galactose and ceramide parts, the presence of aromatic rings or long lipid chains, the installation of a small hydrophobic molecule on the C6 sugar position or a carbocyclic ring instead of the D-galactose moiety. On the other hand, the T_H2 immune response seems to be preferred when one of the lipid chains is truncated, when unsaturations are present in the lipid chains, when the nature of the amide function is modified or when a polar group is fixed in the C6 sugar position. Other analogues have also shown that the presence of double bonds or PEG groups can improve the bioavailability of these compounds. The preparation of several hybrid analogues can be envisaged, but their syntheses will be probably limited by the chemical hurdles encountered (Fig. 15).

For the time being, the relationship between glycolipid structure and cytokine polarisation is not completely understood. Even relatively minor changes in the glycolipid structure, either on the sugar or the ceramide moieties, can result in large affinity differences among the binding partners CD1d and TCR, with a subsequent effect on cytokine production. So behind the structural modifications, scientists must think about the best way to obtain 1) an efficient loading and stability of the glycolipid in CD1d, and 2) a good binding affinity with the TCR to allow a large production of the desired cytokines. Ideally, the biological tests should be preferentially performed on human *i*NKT cells, because the structures of mouse and human *i*NKT cells are quite different. Hypothesizing that stability and binding affinity is related to a specific conformation of the CD1d/glycolipid complex with the particular orientation of some amino acids, it might one day be possible to achieve a T_H1- and T_H2-type conformation of the ternary complex. As we are still far from fully understand all of the mechanisms of the immune system, and as the chemistry can still be improved, the continuing interaction of organic chemists, biologists and chemical physicists[123] is certainly the key to carry out this fabulous "KRN" project. We hope that this review has contributed a clear, complete account of the state of this fast developing field, and help fellow scientists to find new rational hypotheses and clues for their research.

Acknowledgements

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