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3-Fluoro- and 3,3-Difluoro-3,4-dideoxy-KRN7000 Analogues as New Potent Immunostimulator Agents: Total Synthesis and Biological Evaluation in Human Invariant Natural Killer T Cells and Mice

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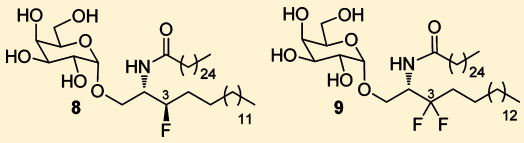
Supporting Information

ABSTRACT: We propose here the synthesis and biological evaluation of 3,4-dideoxy-GalCer derivatives. The absence of the 3- and 4-hydroxyls on the sphingoid base is combined with the introduction of mono or difluoro substituent at C3 (analogues **8** and **9**, respectively) to evaluate their effect on the stability of the ternary CD1d/GalCer/TCR complex which strongly modulate the immune responses. Biological evaluations were performed in vitro on human cells and in vivo in mice and results discussed with support of modeling studies. The fluoro 3,4-dideoxy-GalCer analogues appears as partial agonists compared to KRN7000 for iNKT cell activation, inducing T_H1 or T_H2 biases that strongly depend of the mode of antigen presentation, including human vs mouse differences. We evidenced that if a sole fluorine atom is not able to balance the loss of the 3-OH, the presence of a difluorine group at C3 of the sphingosine can significantly restore human iNKT activation.



INTRODUCTION

α-Linked galactosylceramides (α-GalCers) are a special class of exogenous glycolipid antigens with a potent immunoregulatory effect recognized by the semi-invariant T cell receptors (TCR) of invariant natural killer T cells (iNKT) when presented by the CD1d molecule of antigen presenting cells.[1−3] The trimolecular complex formation initiates a rapid release of pro-inflammatory T helper (T_H1) (e.g., TNF-α, IFN-γ, and IL-2) and anti-inflammatory T helper (T_H2) cytokines (e.g., IL-4, IL-10, and IL-13) by the iNKT cells.[4−7] Disruption of the T_H1/T_H2 balance may lead to disease induction as T_H1 and T_H2 type cytokines can antagonize each other's biological functions.[8] The opposing activities displayed by T_H1 and T_H2 cytokines induced by α-GalCers limit their value as potential therapeutic agents. Production of analogues able to favor either of the two biases without severely altering the immune response is currently the focus of intense research.

Since the discovery of KRN7000, an α-GalCer with a lack of 2′-OH group inducing iNKT activation at nM level[9] (**1**, Chart 1), rationalization of the structure−activity relationship (SAR)[2] has been attempted for several synthetic analogues, facilitated by the availability of the CD1d/KRN7000/TCR trimolecular complex crystal structure.[10,11] Although the stability of the CD1d/GalCer/TCR complex is believed to contribute greatly to the shifting of the immunostimulation profile, numerous factors play a role in the cytokine polarization.

α-GalCers are loaded into the CD1d cleft with only the sugar headgroup exposed for the interaction with the iNKT TCR. The nature of the sugar unit, D-galactose, is strongly preferred,[12] while modifications of the C3 and C6 positions were found to be tolerated.[13−15] Recently, Van Calenbergh and others[16] described the potent antigenic activity of 6″-amido and 6″-ureido derivatives bearing an intact phytoceramide moiety that present a T_H1 orientation in mice. Carbasugar[17] and some open chains mimicking sugar architectures[18,19] also orient the cytokine response with

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Chart 1. Structure of Selected α-GalCers and 4-Deoxy KRN7000 Analogues

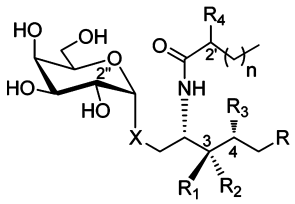
α-GalCer series

R_1, R_3 = OH; R_2, R_4 = H

1 X = O, n = 23, R = $nC_{13}H_{27}$ (**KRN7000**)

2 X = CH_2, n = 23, R = $nC_{13}H_{27}$ (**α-C-KRN7000**)

3 X = O, n = 23, R = nC_4H_9 (**OCH**)



Deoxy α-GalCer analogues

4 X = O, R_1 = OH; R_2, R_3, R_4 = H; n = 23, R = $nC_{12}H_{25}$ (**4-deoxy-KRN7000**)

5 X = O, R_1, R_2, R_3, R_4 = H; n = 23, R = $nC_{12}H_{25}$ (**3,4-dideoxy-KRN7000**)

Deoxy-fluoro-α-GalCer analogues

6 X = O, R_1 = OH; R_2, R_4 = H; R_3 = F, F; R = $nC_{13}H_{27}$ (**4-deoxy-4,4-difluoro-KRN7000**)

7 X = O, R_1 = OH; R_2 = H; R_3 = OH, R_4 = F, F; R = $nC_{13}H_{27}$ (**2',2'-difluoro-KRN7000**)

8 X = O, R_1 = F; R_2, R_3, R_4 = H; R = $nC_{12}H_{25}$ (**3,4-dideoxy-3-fluoro-KRN7000**)

9 X = O, R_1, R_2 = F; R_3, R_4 = H; R = $nC_{13}H_{27}$ (**3,4-dideoxy-3,3-difluoro-KRN7000**)

a T_H1 bias. The α-*O*-anomeric configuration of the ceramide aglycone moiety is believed to be critical[20] as β-galactosylceramides[21,22] display far lower agonist activities, while enhanced T_H1 response in mice is induced by a nonhydrolyzable α-1*C*-galactoside analogue (*C*-KRN7000, **2**, Chart 1).[23] Complementarily, SAR studies focused on the ceramide moiety have concentrated efforts on modifications of the phytosphingosine and acyl chains. Truncation of the sphingosine alkyl chain illustrated by the OCH analogue (**3**, Chart 1), leads to a marked decrease in the IFN-γ/IL-4 ratio in favor of the T_H2 biased immune response.[24,25] On the other hand, aromatic residues on either the acyl or sphingoid base of KRN7000 seem to restore the T_H1 cytokine profile.[26,27]

Throughout all these alterations, one of the remaining features concerns the polarity of the sphingoid base. Concerning its OH groups' influence, we and others[9,28−32] investigated the importance of the 4-OH group in the stimulatory potency of 4-deoxy-KRN7000[33] (**4**, Chart 1). We have recently confirmed its lesser importance compared to that of the 3-OH group as mentioned in some earlier contradictory studies, either in vitro with human cells or in vivo in mice.[9,12,32,34,35] According to previous investigations on 3,4-dideoxy-KRN7000 analogue (**5**, Chart 1),[9,12,34] the sphingoid base 3-OH group is indisputably required for inducing iNKT cell responses.[32] In accordance with these observations, crystallography data have clearly shown that only the key 3-OH group is involved in the stabilization of the trimolecular CD1d/KRN7000/TCR complex by concomitant donor participation with CD1d Asp80 and a suspected acceptor effect with Arg95 of the CDR3α-loop of the TCR.[10,36] In addition, examining the stereochemistry of KRN7000, Chung et al.[37] showed that the relative spatial orientations of 2-NH and 3-OH groups are both important for activity, with a greater impact for the configuration of 2-NH.

More recently, fluorine atom(s),[38] that imparts various properties to certain drugs, affecting binding interactions, metabolic stability, and selective reactivities, has been introduced into α-GalCer structures, leading to alterations on cytokine releases. Linclau et al.[39] reported the synthesis of a 4-deoxy-4,4-difluoro-KRN7000 analogue (**6**, Chart 1) in which the hydrogen-bond donating capacity with CD1d was reinforced vs a concomitant decrease in its ability to accept the hydrogen bond from the TCR. In vivo evaluation of the latter *gem*-difluoro analogue resulted in a minimal loss in IFN-γ secretion compared to KRN7000. The authors concluded that a tighter bonding of the 3-OH group with CD1d is beneficial for a T_H1 polarization, while the hydrogen bond with the TCR seems of lesser importance. The same authors also reported biological evaluation of 2′,2′-difluoro KRN7000[40] (**7**, Chart 1) in which the acidity of N−H amide group of the ceramide fragment should be increased by introduction of the two neighboring difluorine atoms. While expecting to favor a T_H1 bias by reinforced hydrogen bonding with Thr156 of mCD1d (Thr154 for hCD1d) to stabilize the CD1d/GalCer/TCR complex, a surprising T_H2 bias was observed. The authors suggested that the latter unexpected polarization of the immune response indicates that hydrogen bonding between the amide and the TCR receptor does not have a significant impact on the stability of the ternary complex.

In our continuing efforts to highlight the potency of deoxy analogues of α-GalCers to modulate the iNKT response, we evaluated the effect of combined alterations of the sphingoid base devoid of both 4-OH and 3-OH groups by substituting the 3-OH groups by one or two fluorine atoms (3,4-dideoxy-3-fluoro-KRN7000 **8** and 3,4-dideoxy-3,3-difluoro-KRN7000 **9**, respectively, Chart 1). Our aim was to contribute to establish the relative part of the TCR interaction with the 3-OH group of α-GalCers. We anticipated that, despite suppression of the hydrogen-bond donating capacity with CD1d, electron isoelectronic effect of one or two fluorine atoms, vs oxygen, would modulate the lack of the sphingosine 3-OH on the destabilization of the CD1d/GalCer/TCR complex by reinstating favorable interaction with the TCR.

RESULTS

Synthesis of 3,4-Dideoxy-3-fluoro-KRN7000 8. We have recently reported an access to a 4-deoxy-KRN7000 analogue (**4**, Chart 1) from the key ethylenic galactoside intermediate **11** (Scheme 1).[30] This latter derivative was readily obtained by coupling of the α-fluoro-perbenzylglycosyl donor **12** with the acceptor sphingosine fragment **13**. Cross-coupling metathesis reaction between the glycosidic primer **11** and a corresponding ethylenic partner gave the sphingosine intermediate **10** (R_1 = *n*-$C_{12}H_{25}$). The last step, prior to total deprotection of

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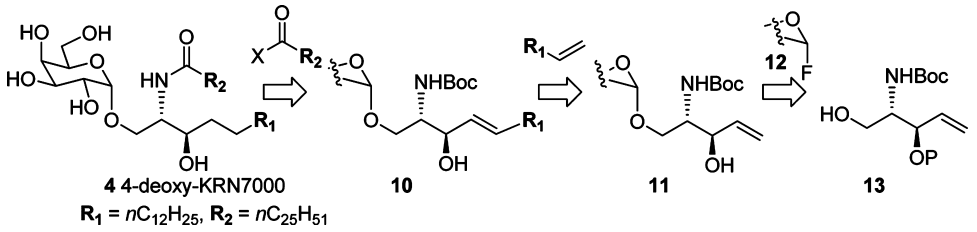
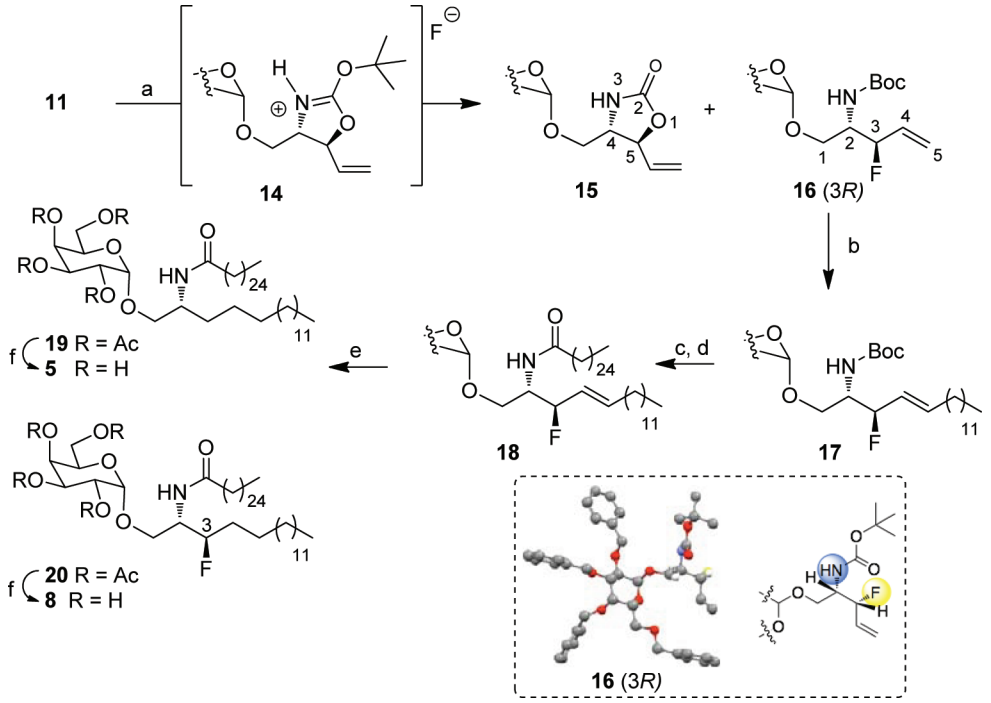
Scheme 1. Retrosynthetic Pathway to 4-Deoxy-KRN7000 4



Scheme 2. Synthesis of 3,4-Dideoxy-3-fluoro-KRN7000 4 and RX Structure of 16[a]



[a]Reagents and conditions: (a) DAST, CH_2Cl_2, 10 min, rt, **16** 54%, **15** 35%; (b) 1-tetradecene, Grubbs II catalyst, CH_2Cl_2, reflux, 24 h, 83%; (c) HCl 2N solution in Et_2O, THF, reflux; (d) $nC_{25}H_{51}COCl$, 50% aq NaOAc, THF, 40 °C, 1 h, 38% over two steps; (e) H_2, Pd/C, $CHCl_3$/MeOH, 48 h, rt, then Ac_2O/pyridine, DMAP, separation by flash chromatography on silica gel **19** 45%, **20** 29%; (f) MeONa, CH_2Cl_2/MeOH (1:1), for **5** 47% and for **8** 52%.

the target, consisted of an acylation of the free amine group to complete the ceramide moiety of the 4-deoxy-KRN7000.

In the light of this previous work, we planed to synthesize the 3,4-dideoxy-3-fluoro-KRN7000 derivative **8** via the ethylenic 3-deoxy-3-fluoro-galactoside **16** as outlined in Scheme 2. The fluorine substituent was expected to be introduced in the required configuration (3*R*) from the allylic alcohol **11** following a double S_N2 inversion of configuration. Thus, fluorination of **11**, by DAST at −78 °C in CH_2Cl_2,[41] promoted the formation of the expected fluoro derivative **16**, in moderate 30% yield, along with the oxazolidinone byproduct **15** formed predominantly (60%). The absolute configuration 3*R* of fluoro derivative **16** was unambiguously assigned at solid state by X-ray crystallography (see Supporting Information for selected data). After 3-OH activation by DAST, the NHBoc neighboring group cyclized,[42] giving rise to intramolecular S_N2 formation of an oxazolidinium salt **14**. Subsequent nucleophilic addition of the released fluoride anion to the iminium **14** provided the fluorinated derivative **16** (3*R*) upon a second inversion of configuration at the C-3 stereogenic center. Nevertheless, a favorable loss of isobutene residue from **14**, via the release of stable *tert*-butyl carbocation, from **14** led to the major formation of the undesired oxazolidinone **15** (4*S*). The latter side reaction can be minimized by running the fluorination reaction at higher temperature (rt vs −78 °C), yielding the desired fluorinated intermediate **16** in an optimized 54% yield, along with a minor amount of **15** (35%). At this stage, Grubbs II catalyzed cross-metathesis reaction between the fluoro allyl **16** and 1-tetradecene provided access to the 3-fluorinated sphingosine **17** in 83% yield (Scheme 2). After acidic removal of the *N*-Boc group, the acylation of sphingosine fragment, following a well-established method described in GalCers series (4-nitrophenyl hexacosanoate, in the presence of Et_3N and catalytic DMAP), failed to give the expected ceramide derivative **18** from **17**. A probable electronic deactivation by the vicinal fluorine atom should be taken into account to explain the loss in reactivity of the amine partner. Eventually, success was encountered by using *N*-acylation conditions described by De Jonghe[43] and Teare[44] for the synthesis of fluorinated sphinganine and sphingosine, respectively. The crude mixture, obtained after cleavage of the *N*-Boc protecting group from **17**, was treated with hexacosanoyl chloride in 50% NaOAc aqueous solution, at 40 °C, to give the *N*-acylated derivative **18** in a modest 38% yield. Final

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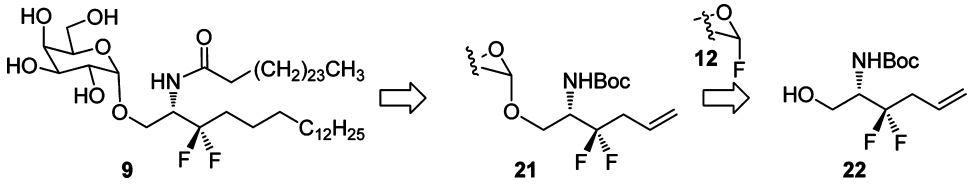
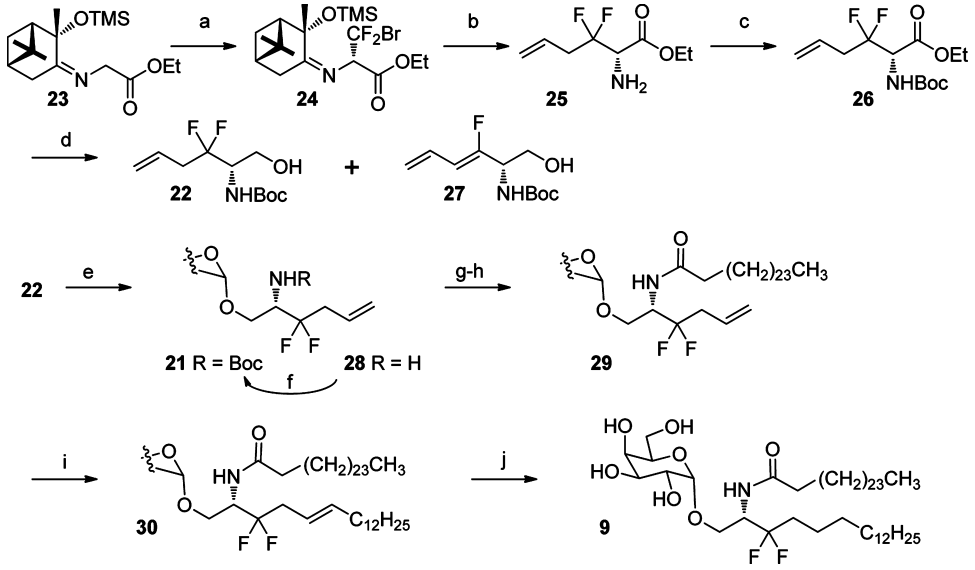
Scheme 3. Retrosynthetic Scheme for the Synthesis of 3,3-*gem*-Difluoro Analogue 9



Scheme 4. Synthesis of 3,4-Dideoxy-3,3-difluoro Analogue 9[a]



[a]Reagents and conditions: (a) LiHMDS, THF, −80 °C, 30 min, then CF_2Br_2, −100 °C, 1 h, 53% after recrystallization; (b) (i) allyltri-*n*-butyltin, AIBN cat., benzene, reflux, 3 h, (ii) EtOH/HCl 2N (9:1), 50 °C, 1 h; (c) Boc_2O, $NaHCO_3$, THF, rt, overnight, 39% (over 3 steps); (d) $NaBH_4$, EtOH, 0 °C to rt, 4 h, **22** 81% and **27** <5%; (e) **12**, $SnCl_2$, $AgClO_4$, THF/Et_2O (1:3), 0 °C, 1 h then rt, 1 h; (f) Boc_2O, $NaHCO_3$, dioxane, **21** 40% over 2 steps; (g) HCl gas, Et_2O, rt, 3 h; (h) $nC_{25}H_{51}COCl$, pyridine, 80 °C, 24 h, 38%; (i) 1-tetradecene, Grubbs II catalyst, CH_2Cl_2, reflux, 4 h, 45%; (j) $Pd(OH)_2/C$, H_2, $CHCl_3$/EtOH (1.5:1), 40%.

hydrogenolysis of the benzyl protecting groups with Pd/C and concomitant hydrogenation of the unsaturation, achieved the synthesis of the targeted 3,4-dideoxy-3-fluoro-KRN7000 **8**. In the latter conditions, an equimolar amount of the byproduct, 3,4-dideoxy derivative **5**, was formed when Pd or $Pd(OH)_2$ on carbon catalysts were used. The sensitivity of the allylic fluorine atom to hydrogenolysis conditions has been also observed by Hsin[45] and Hudlicky.[46] Because compounds **8** and **5** are inseparable by chromatography on silica gel, their purification was achieved via acetylation of the crude hydrogenation mixture (Ac_2O/pyridine 1:2, and DMAP cat.), giving the peracetylated derivatives **19** and **20** that have been separated by flash chromatography in 45% and 29% yields, respectively. Deprotection of the peracetylated intermediates by sodium methanolate in methanol gave the expected 3,4-dideoxy-3-fluoro-KRN7000 **8** and 3,4-dideoxy-KRN7000 **5** in 52% and 47% yields, from **20** and **19**, respectively.

Synthesis of 3,4-Dideoxy-3,3-difluoro-KRN7000 9. To supplement our investigation on the impact of a fluorine atom in place of 3-OH group, the synthesis of 3,4-dideoxy-3,3-difluoro KRN7000 **9** was undertaken. To be faithful to our synthetic strategy, the preparation of *gem*-difluoro analogue **9** relies on the building of a 3,3-*gem*-difluorinated ethylenic galactoside fragment **21** to be engaged in a cross-metathesis process (Scheme 3).

The required homoallylic *gem*-difluoro alcohol **22** could be obtained via sulfoxide chemistry[47] or following the five-step procedure described by Katagiri et al.:[48] alkylation of ethyl glycinate Schiff's base **23** with CF_2Br_2, radical allylation of bromide **24** followed by hydrolysis of the chiral group, and *N*-Boc protection of the resulting amine **25** prior reduction of corresponding ester **26** (Scheme 4). The latter procedure was retained as (2*S*)-ethyl-2-*tert*-butoxycarbonylamino-hex-5-enoate **26** has been already used for other chemistry in our group. The reduction of ester **26** into alcohol **22**, by $NaBH_4$ in EtOH, proceeded without major difficulties in good 81% yield, although some HF elimination product **27** (that could not be separated from the alcohol **22**) was formed concomitantly. However, its formation could be kept minimal (<5%) by controlling carefully the temperature and the reaction time.

The reaction sequence was then pursued from the alcohol **22** by a glycosylation under Mukaiyama conditions in the presence of fluorogalactosyl donor **12**. The reaction led to a mixture of protected *N*-Boc protected and free NH_2 α-galactosides **21** and **28**, isolated in low 10% and 25% yields, respectively, after purification. The amine derivative **28** was quite difficult to purify, and flash chromatography led to a loss of material. Consequently, the crude mixture of the glycosidic coupling reaction was treated under Boc protection conditions (Boc_2O, $NaHCO_3$) to give the *gem*-difluoro-α-galactoside **21** in 40% yield over the two steps. Then *N*-Boc group was removed by HCl_g in Et_2O, and the resulting crude amine **28** was directly engaged in the next step without purification. Introduction of the hexacosanoyl acyl chain was first attempted from

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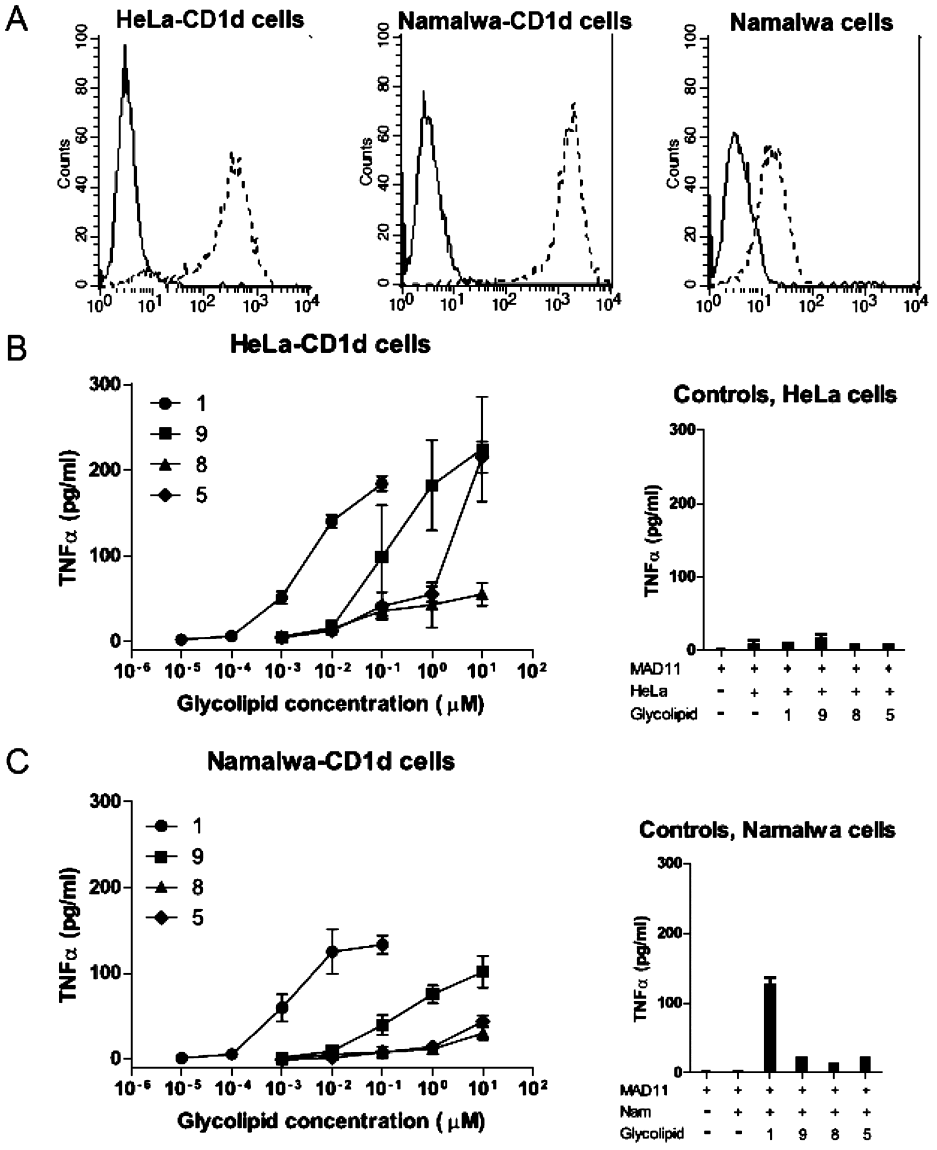

Figure 1. (A) Flow cytometric analysis of hCD1d-transduced HeLa cells, CD1d-transduced and nontransduced Namalwa cells. Gray lines represent CD1d expression detected by anti-CD1d-PE mAb 51.1 and black lines represent controls where only the secondary antihuman IgG Ab was used. A total of 250000 cells were analyzed in each experiment. The figure is representative of at least 3 separate experiments. (B and C) Relative potencies of compound **1** (KRN7000, represented by circles) and analogues **9** (3,4-dideoxy-3,3-difluoro KRN, represented by squares), **8** (3,4-dideoxy-3-fluoro KRN, represented by triangles), and **5** (3,4-dideoxy KRN, represented by diamonds) to induce TNFα production by the Vα24 iNKT cell line MAD11. The CD1d-transduced antigen-presenting cell lines HeLa (B) and Namalwa (C) were pulsed with increasing concentrations of glycolipids and iNKT cells were stimulated for 6 h. Background values obtained when no exogenous glycolipid was added to the CD1d-transduced antigen-presenting cells were subtracted from the presented values. Negative controls with MAD11 cells alone and CD1d nontransduced antigen presenting cells in the presence and absence of the glycolipid compounds **1**, **9**, **8**, and **5** are shown on the right-hand side. TNFα secretion was measured by a cellular assay, and results are shown as mean of triplicates from one representative experiment out of at least three with similar results. Error bars represent mean values ± SD.

hexacosanoyl chloride (n-$C_{25}H_{51}COCl$, NaOAc, THF/H_2O, 45 °C) or from hexacosanoic acid (EDC, NMM, HOBt, CH_2Cl_2, rt), affording only trace of the expected acylated product **29**. Similarly, the reaction of **28** in the presence of hexacosanoyl chloride in pyridine at 50 °C proceeded extremely slowly, and the expected product **29** was obtained in a poor 6% yield. However, heating the reaction mixture at 80 °C for 24 h increased the yield of the acylation to 36%. Ruthenium-catalyzed cross-metathesis reaction between 1-tetradecene and homoallylic *gem*-difluoro-compound **29**, under the same conditions as for preparation of compound **17** (see Scheme 2), led to the desired unsaturated compound **30** isolated in 45% yield after purification. Finally, treatment of the intermediate **30** under hydrogen atmosphere in the presence of $Pd(OH)_2/C$ performed the subsequent hydrogenolysis of the benzyl protective groups and the reduction of the double bond to give 3,4-dideoxy-3,3-difluoro-KRN7000 **9** in 40% yield.

Biological Evaluations. *In Vitro Results.* The ability of the 3,4-dideoxy analogues to activate iNKT cells in vitro was investigated using two antigen-presenting cell lines transduced to express hCD1d. FACS analysis verified that the two cell types expressed comparable levels of hCD1d after transduction (Figure 1A). HeLa cells of epithelial origin are spontaneously CD1d negative (result not shown), whereas Namalwa cells of

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leucocytic origin spontaneously express low levels of CD1d. Parts B and C of Figure 1 show the TNF-α secretion by the iNKT MAD11 cell line after stimulation with antigen-presenting cells pulsed with compound **1** (KRN7000) and the dideoxy α-GalCer analogues **5** (3,4-dideoxy KRN7000), **8** (3,4-dideoxy-3-fluoro KRN7000), and **9** (3,4-dideoxy-3,3′-difluoro KRN7000). We have previously shown that the 4-deoxy analogue of KRN7000 stimulates iNKT cells nearly to the same extent as reference **1**.[30] However, as shown in Figure 1, removal of the hydroxyl groups at both C3 and C4 in the 3,4-dideoxy KRN7000 analogue **5** renders it a poor stimulator of human iNKT cells and lowers its stimulatory effect by a 1000-fold when presented by HeLa-CD1d cells (Figure 1B, left) and >10000-fold when using Namalwa-CD1d cells (Figure 1C, left). Addition of a fluorine atom at C3 in monofluoro derivative **8** further lowers the activity >10000 times compared to **1**, using both types of antigen-presenting cell lines (Figure 1B and C, left). Intriguingly, addition of a second fluorine atom at C3 in 3,3-difluoro KRN7000 analogue **9** partly restores the activity with a stimulatory effect only 20 times lower than that of compound **1** when presented by HeLa-CD1d cells (Figure 1B, left) and 100 times lower when presented by Namalwa-CD1d cells (Figure 1C, left). These results indicate that the loss of the hydroxyl group at C3 in the sphingoid base of analogues **5** and **8** destabilizes the CD1d/glycolipid/TCR complex, as expected. In contrast, the presence of two geminal fluorine atoms in the 3,3-difluoro analogue **9** restabilizes the complex. As a control, all glycolipids were also loaded onto nontransduced HeLa and Namalwa cells in order to confirm that they were presented by CD1d. Glycolipids **1**, **5**, **8**, and **9** loaded onto HeLa cells induced only background TNF-α secretion (Figure 1B, right). When loaded onto Namalwa cells (Figure 1C, right), TNF-α release was about 20−90% of the secretion obtained when loaded to CD1d-transduced cells, in agreement with the FACS results showing significant native expression of CD1d in Namalwa cells (Figure 1A).

To determine if stimulation by the 3,4-dideoxy analogues led to a bias in the T_H1/T_H2 balance of the response, we measured the secretion of a T_H1 type (IFN-γ) and a T_H2 type (IL-13) cytokines. IL-13 was chosen because IL-4 secretion was always extremely low in our in vitro assay conditions. HeLa and Namalwa cell lines were pulsed with increasing concentrations of glycolipids, and iNKT cells were stimulated for 6 and 48 h for the IFN-γ and IL-13 analysis, respectively. Figure 2 shows the ratio between the releases of the T_H1 type cytokine IFN-γ (left y axis, solid line) and the T_H2 type cytokine IL-13 (right y axis, dotted line) induced by the glycosylceramides **1**, **5**, **8**, and **9**. Because compound **1**, KRN7000, is taken as the reference for the T_H1/T_H2 balance (ratio IFN-γ/IL-13 = 1), the curves illustrating the release of IFN-γ and IL-13 induced by this glycolipid were superimposed and compared to the super-imposed curves generated by the dideoxy analogues in order to establish their T_H1/T_H2 profiles. When presented by HeLa-CD1d cells (Figure 2A−C, left), analogues **5**, **8**, and **9** all generated IFN-γ/IL-13 relative secretion similar to that obtained by compound **1**, and intensities of the responses for each of these cytokines paralleled those obtained for TNF-α. In contrast, when Namalwa-CD1d cells were used as antigen-presenting cells, 3,3-difluoro analogue **9** gave an IFN-γ/IL-13 profile identical to that of **1**, whereas analogues **8** and **5** appeared to induce slightly elevated levels of IL-13 relative to IFN-γ indicative of a weak T_H2 orientation by comparison with **1** (Figure 2A−C, right). However, regardless of the glycolipid, IL-13 secretion was always weaker upon presentation by Namalwa-CD1d cells than by HeLa-CD1d cells (note that the scales for IL-13 are different between the left and right panels of Figure 2). In addition, no difference in IL-13 secretion was noted between compounds **9**, **8**, and **5** when presented by Namalwa-CD1d, suggesting a distinct behavior depending on the antigen presenting cells. The factors that determine whether an immunologic challenge will trigger a T_H1 or a T_H2 response are not fully understood, but a correlation has been established between the stability and interaction time of the CD1d/glycolipid/TCR complex and the type of cytokines that are being secreted. A more stable complex allows for a longer interaction and secretion of T_H1 type cytokines, whereas a destabilized complex leads to a shorter interaction time and release of T_H2 cytokines. Our results show a slight T_H2 orientation compared to reference compound **1** for analogues 3,4-dideoxy-3-fluoro KRN7000 (**8**) and the 3,4-dideoxy KRN7000 (**5**), visible upon presentation by Namalwa-CD1d cells. This is in agreement with the results obtained in the TNF-α analysis (above), i.e., that the loss of the hydroxyl H-donating group at C3 in the long chain base destabilizes the CD1d complex, while addition of a second fluorine atom in *gem*-difluoro analogue **9** increases the complex stability. However, the differences observed in terms of IL-13 secretion between presentation by either the HeLa-CD1d or Namalwa-CD1d cells indicate that other factors related to the presenting cells contribute to dictate the response orientation. The negative controls in Figure 2D (right charts) confirm that, when loaded to non-CD1d transduced HeLa cells, the glycolipids induced only background quantities of IFN-γ and IL-13 release. When loaded to Namalwa cells, the cytokine releases were about 0−30% (IFN-γ) and 10−40% (IL-13) of the secretion obtained when loaded to CD1d-transduced cells, in agreement with the FACS analysis shown in Figure 1A.

In Vivo Results. To investigate whether 3,4-dideoxy-KRN7000 analogues **5**, **8**, and **9** could elicit CD1d-dependent NKT cell activation in vivo, mice received intraperitoneally (ip) 100 μg/kg of glycolipids in 0.1 mL of vehicle after which TNF-α, IFN-γ, and IL-4 kinetic releases were measured and compared to the secretion induced by KRN7000. Injection of 3,4-dideoxy-3-fluoro **8** and 3,4-dideoxy-3,3-difluoro **9** KRN7000 analogues resulted in notably lower serum cytokine levels relative to KRN7000. The area under curve (AUC) for TNF-α release induced by both fluorine derivatives **8** and **9** appeared quite similar, arising at 23% and 32% of KRN7000 potency. Intriguingly, the 3,4-dideoxy-derivative **5** promoted TNF-α release with a 40% relative efficiency (Figure 3a). This behavior was highlighted by the INF-γ cytokine levels induced by compound **5**, providing an AUC over 70% close to that of KRN7000 (Figure 3b). The difluorinated analogue **9** induced a noticeable in vivo INF-γ stimulation only twice lower than that of KRN7000, 8 h after injection, while that of monofluorinated compound **8** leveled off at a maximum of 30%. IL-4 amounts induced by all dideoxy-analogues **5**, **8**, and **9** appeared quite similar (Figure 3c), providing AUC dideoxy analogues/KRN7000 ratio ranging between 0.35 and 0.39. Consequently, a T_H1 orientation was observed with the 3,4-dideoxy-3,3-difluoro-KRN7000 **9** and more surprisingly also with the 3,4-dideoxy-KRN7000 **5**, providing AUC IFN-γ/IL-4 ratio of 1.31 and 1.86, respectively (Figure 4). In contrast, mono-fluorinated derivative **8**, that stimulated a 3-fold lower release of IFN-γ than KRN7000, favored a slight T_H2 profile with an AUC IFN-γ/IL-4 ratio of 0.78. These results clearly indicate

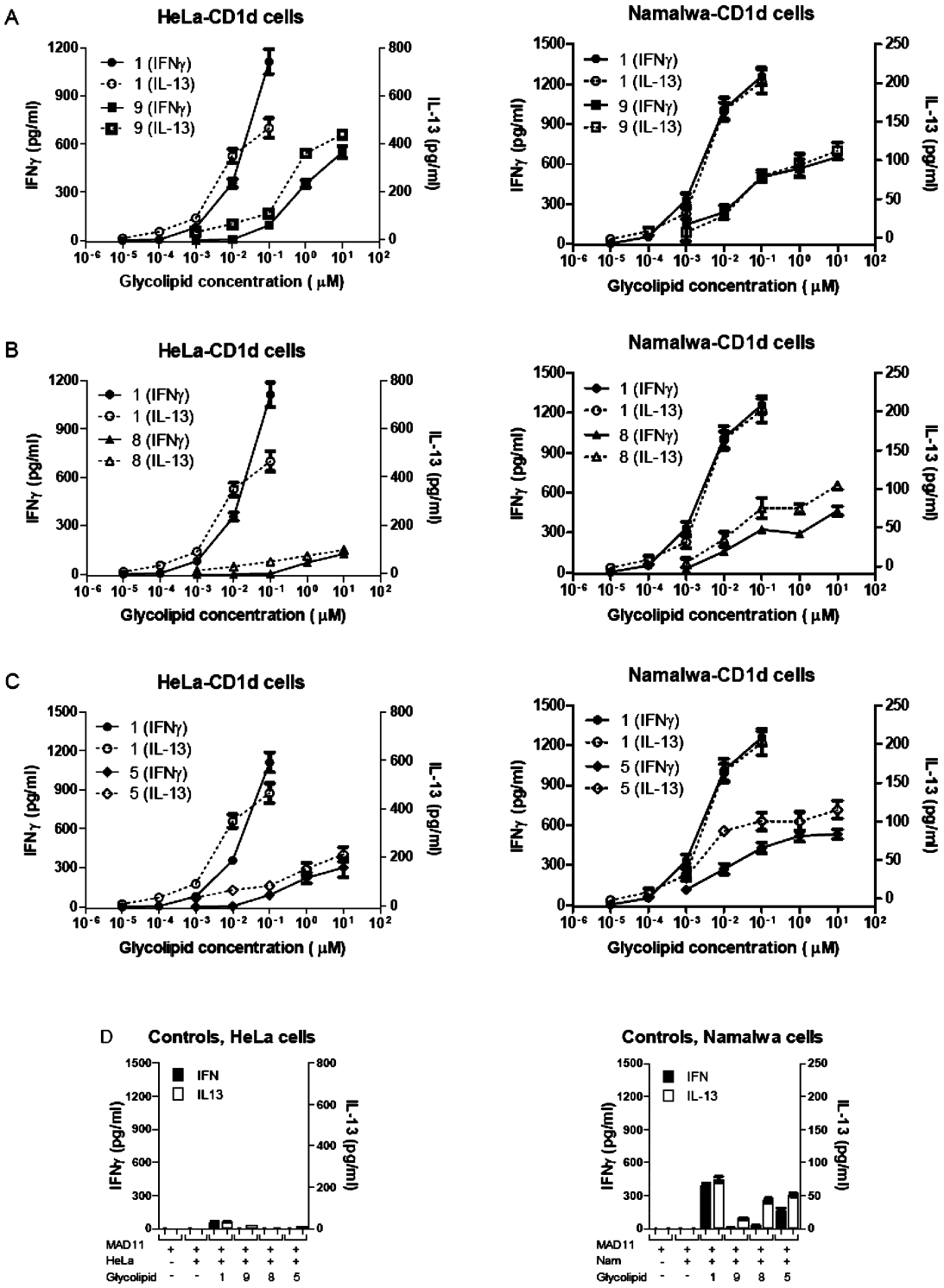

Figure 2. Relative potencies of compound **1** (KRN7000, represented by circles) and analogues **9** (3,4-dideoxy-3,3-difluoro KRN, represented by squares), **8** (3,4-dideoxy-3-fluoro KRN, represented by triangles), and **5** (3,4-dideoxy KRN, represented by diamonds) to induce secretion of IFNγ (left *y*-axis, represented by solid lines and filled symbols) and IL-13 (right *y*-axis, represented by dotted lines and open symbols) by the Vα24 iNKT cell line MAD11. The CD1d-transduced antigen-presenting cell lines HeLa (left-hand side) and Namalwa (right-hand side) were pulsed with increasing concentrations of glycolipids and iNKT cells were stimulated for 6 and 48 h for the IFNγ and IL-13 analysis, respectively. Compound **1** is shown in all graphs A–C as a T_H1/T_H2 neutral reference glycolipid. Background values obtained when no exogenous glycolipid was added to the CD1d-transduced antigen-presenting cells were subtracted from the presented values. Negative controls with MAD11 cells alone and CD1d nontransduced antigen presenting cells in the presence and absence of the glycolipid compounds **1**, **9**, **8**, and **5** are shown in (D). Cytokine secretion was measured by ELISA, and results are shown as mean of triplicates. Error bars represent mean values ± SD.

that the behavior of the 3,4-dideoxy compounds differ between the in vivo assay in mice and the in vitro assay with human cells, the most notable difference being observed for compound **5**.

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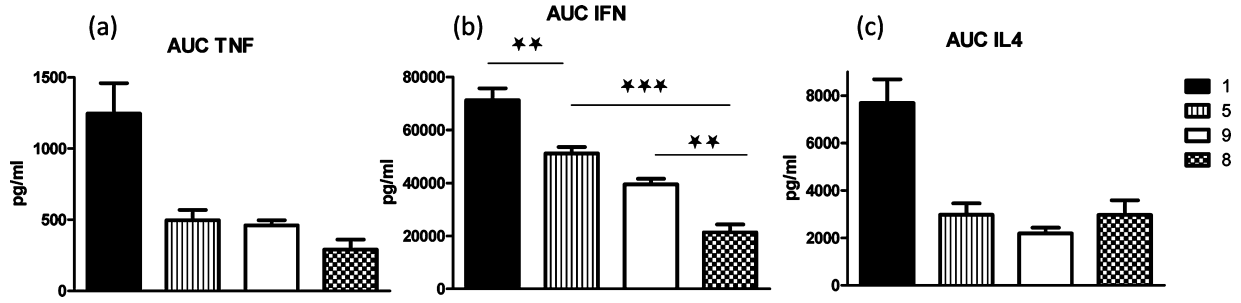

Figure 3. AUC TNF-α (a), IFN-γ (b), and IL-4 (c) levels in C57BL/6J Rj mice serum after ip injection of Galcers **1**, **8**, **9**, and **5**. Compound **1** always stimulates significantly higher levels of cytokines than the other compounds ($p < 0.001$ for all, except for IFN level of compound **5** ($p < 0.01$)). $p < 0.01$ = **, $p < 0.001$= ***.

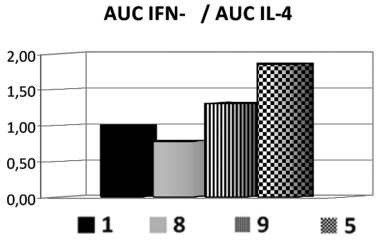

Figure 4. AUC IFN-γ/AUC IL-4 ratio in C57BL/6J mice serum after ip injection of GalCers **1**, **8**, **9**, and **5**.

DISCUSSION

Elimination of the CD1d Asp80 hydrogen bond by replacing the 3-OH by a fluorine atom in α-GalCer structures drastically reduced the magnitude of cytokine releases, both in mice and human iNKT cells. The weak stimulation induced by the 3,4-dideoxy-3-fluoro KRN7000 analogue **8** was consistent with the established crucial role of the donating hydrogen bond that stabilizes the CD1d/GalCer complex.[24,49−52] Consequently, the slight T_H2 profile of 3,4-dideoxy-3-fluoro analogue **8**, observed either in vitro on human iNKT cells (IL-13 vs IFN-γ when presented by Namalwa-CD1d cells) and in mice (AUC IFN-γ/IL-4 ratio of 0.78), was anticipated due to a predictable destabilization of the long chain base in the (C′)F′-binding groove. Nevertheless, intriguing effects were observed when two geminal fluorine atoms were introduced at the C3 position of the sphingosine chain. The agonist activity on the TNF-α release of 3,4-dideoxy-3,3-difluoro-KRN7000 **9** appeared only 20- and 100-fold lower than that of KRN7000 **1** on human iNKT cells when presented by either HeLa-CD1d and Namalwa-CD1d cells, respectively. Furthermore, difluoro analogue **9** expressed an IFN-γ/IL-13 profile identical to that of KRN7000 when loaded on Namalwa-CD1d cells. This outcome was pronounced in vivo, inducing a TNF-α secretion in mice only twice lower than that of KRN7000, with a slight T_H1 polarization (AUC IFN-γ/IL-4 ratio = 1.31).

Several interpretations can be made from these observations, taking into account a combination between the close isosteric relationship of fluorine atom to hydroxyl and the strong withdrawing electronic induction. It is well-known that addition of fluorine atom(s) on carbon induces a drastic increase of acidity of a vicinal acidic group (pK = 4.76, 2.59, and 1.24 for CH_3COOH, CH_2FCOOH, and CHF_2COOH, respectively). Obviously, such an electronic effect can be transposed to the ceramide NH−CO bound to contribute in the CD1d/GalCer/TCR interaction. Thus, the destabilization of the ternary complex, due to the lack of the donating 3-OH hydrogen bond with the CD1d, was thought to be offset by reinforcing the donating NH-bond of the group potentially involved in a TCR interaction.[13] The existence of such a TCR−HNCO bond has emerged from X-ray crystallographic studies[36] through a critical interaction with adjacent Thr residue (Thr156 for mCD1d and Thr154 for hCD1d), without involvement of the amide oxygen in CD1d. Consequently, on the basis of the hydrogen bond arguments, the increased acidity of the NH-amide induced by the withdrawing effect of fluorine activation would bias the iNKT cells response toward a T_H1 orientation by restabilizing the GalCer/TCR complex or, at least, would favor selected cytokine secretion. In contrast with this prediction, Linclau et al. have found that introduction of a *gem* 2′,2′-difluorine group on the acyl chain of KRN7000 (compound **7**, Chart 1) elicits a T_H2 polarization in mice.[40] Considering a small conformational impact of a gem difluorine group, relative to an OH group, the authors have correlated this outcome to a negative impact of the modified acyl chain on the polar group stabilization, consistent with a lower affinity with the TCR, rather than an effect of the amide NH···mice Thr156 bond. Obviously, the cytokine secretion restored by difluoro analogue **9** gives a better insight into the amide network by delivering more effective inductive electronic effects from the 3,3-*gem* difluoro group through the $F_2C3−C2−NHC1$ linkage.

A hybrid QM/QM′ model (see Supporting Information for computational details) has been applied for the fluoro KRN7000 analogues to quantify the strength of the hydrogen-bond interaction with Thr154. Because we are only interested in relative variations, a rather small model was defined, including the KRN7000 analogue and seven AA residues (Figure 5) but allowing full ab initio calculations. The results are summarized in

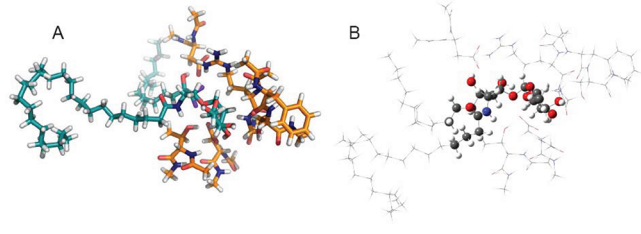

Figure 5. (A) Partially optimized structure of a model of **9** in interaction with seven AA residues of the protein at the PBE0/6-311G(d,p):PBE0/6-31G level of theory. Red atom = O, dark-blue atom = N, purple atom = F, light-blue atom = C of the ligand, orange atom = C of protein residue. (B) Model system investigated: in ball-and-stick the QM part [level of theory: PBE0/6-311G(d,p)], in wireframe the QM′ part [level of theory: PBE0/6-31G]. Red atom = O, blue atom = N, gray atom = C.

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Table 1 for the above-mentioned amide NH-bond, the fluorine-free structure **1** being used as reference. First, it should be noted

Table 1. Theoretical Results[a]

groups	model of	$d_{H\ (NH\text{-}Thr154)}$	q_H	q_O	q_N	$E^{(2)}_{n\rightarrow\sigma^*}$
R_1 = OH; R_2 = R_4 = H	**1**	1.819	0.07	−0.46	−0.66	8.23
R_1 = F; R_2 = R_4 = H	**8**	1.813	0.08	−0.46	−0.62	8.49
R_1 = R_2= F; R_4 = H	**9**	1.818	0.19	−0.52	−0.69	8.69
R_1 = OH; R_2 = H; R_4 = F, F	**7**	1.842	0.10	−0.44	−0.44	7.75

[a] d_H is the amide N-hydrogen-bond distance (in Å), q are the partial atomic MK charges (in |e|) computed for the three atoms implied in the α-GalCer analogues−Thr154 hydrogen bond, and $E^{(2)}_{n\rightarrow\sigma^*}$ are the averaged $n \rightarrow \sigma^*$ interaction energies (in kcal·mol^{-1}) that characterize the H-bond strength. See Chart 1 for substitution positions (in all these models X = O, R_3 = OH, and the sphingoid base contains 18 carbon atoms).

that in models **1**, **8**, and **9**, neither the hydrogen-bond distances nor the atomic charges are strongly affected by the substitution with fluorine atoms on the C3 carbon atom. Therefore, we predict no drastic modifications of the nature of the interaction. Nevertheless, it appears that a 2′-CH_2 substitution by CF_2 on the acyl chain of **7** implies a less negative nitrogen atom (−0.44e instead of −0.66e) accompanied by a lengthening of the hydrogen bond (ca. +0.02 Å). Considering the nearly constant charge of the shared hydrogen atom, this elongation suggests a less favorable interaction between the two moieties, as confirmed by the smaller $E^{(2)}_{n\rightarrow\sigma^*}$ value between the oxygen lone pair $n_{(O)}$ and the unoccupied antibonding orbital $\sigma^*_{(N-H)}$ of the amide moiety. Despite the presence of two electron-withdrawing fluorine atoms, the hydrogen-bond interaction with Thr154 is therefore slightly decreased in the analogue **7**, a finding consistent with its T_H2 profile. This behavior actually originates in the weak intramolecular interaction taking place between an occupied nonbonding orbital of one fluorine atom, $n_{(F)}$ and the unoccupied antibonding orbital $\sigma^*_{(N-H)}$ of the amide moiety. Our model yields a $E^{(2)}_{n\rightarrow\sigma^*}$ value of 0.40 kcal mol^{-1} for this intramolecular interaction (data not shown) that does not occur in the nonfluorinated analogue **1** nor in the monofluorinated **8** where the fluorine and the NH moieties are spatially distant. For the 3,3-difluoro analogue **9**, the second fluorine atom is favorably oriented toward the NH bond but the $E^{(2)}_{n\rightarrow\sigma^*}$ value is significantly smaller (0.13 kcal mol^{-1}), hinting that the intermolecular interaction with Thr154 is not weakened for this difluorinated derivative. Theory therefore foresees for **9** similar geometrical parameters as in KRN7000 **1** but a twice more positive amide hydrogen, implying a stronger donating interaction with Thr154. This assumption is consistent with the larger $E^{(2)}_{n\rightarrow\sigma^*}$ energy reported in Table 1. Eventually, the features of **8** are very similar to those of the reference molecule **1**, and one can assume a relatively trifling impact of this substitution on the NHCO/TCR participation. Although the results of such simulations should certainly not be considered blindly, they seem to indicate a positive relationship between the strength of the difluoro KRN7000 analogue **9**−Thr154 hydrogen bond of the TCR and the experimental findings, i.e., the T_H1 bias of **9** in mice or the clear recovery of cytokine secretion of human iNKT cells.

Thus, modeling studies confirm that positioning the modified C3 difluoro analogue **9** in the A′ pocket of hCD1d exposes the 3(*S*)-fluorine atom in a *syn*-periplanar conformation to the NH amide group (3(*S*)F−C3−N−H: 0.6°, Figure 5), allowing an optimum electron withdrawing effect.

An additional aspect should be also discussed concerning a possible C_3−F···H−Arg95 hydrogen bond participation in the complex stabilization, as hydrogen bonds involving fluorine atom have emerged from several protein structures cocrystallized with fluorine-containing ligands.[53] Even if C−F···H−O bond energy was measured at less than half the strength of a C−O···H−O bond (between 2 and 3.38 kcal mol^{-1}), consistent interaction can occur when the fluorine atom possessed a short C−F···H− contact of ≤2.35 Å.[54,55] Thus interaction of the fluorine atom pointing toward the CDR3α loop of the TCR from the sphingoid base can be envisaged with respect to CD1d/**9**/TCR modeling data, giving the C3(*S*)-F···H−Arg95 and C3(*R*)−F···H−Arg95 distances at 2.286 and 2.292 Å, respectively. Both are closer than the 3-HO···H distance in reference **1** measured at 2.471 Å. However, as confirmed in vitro by the cytokine release levels using 3,4-dideoxy-3(*R*)-fluoro KRN7000 analogue **8**, this interaction does not balance the lack of the donating hydrogen bond from C3-OH group. The weak stimulatory potency of 3(*R*)-fluoro compound **8** allowed also to eliminate the occurrence of a double acceptor binding by the same fluorine atom in the ternary complex network that could involve two H-donor groups.[53]

Along this discussion, it is intriguing that 3,4-dideoxy-KRN7000 analogue **5**, used as negative control, promotes TNF-α and INF-γ releases in mice with 40% and 70% respective efficiencies relative to KRN7000 in spite of the absence of both hydroxyl groups on the sphingoid base. These data are consistent with previous works,[9,25] that regarded the 3,4-dideoxy-KRN7000 **5** as a partial agonist in mice in spite of a nonstimulatory effect on human cells in vitro. Thus, despite a pronounced T_H1 polarization by 3,4-dideoxy-KRN7000 **5** in mice (AUC IFN-γ/IL-4 ratio of 1.86), this behavior was definitively not observed with human cells. Additionally, Sidobre et al.[32] have noted the poor potency of 3,4-dideoxy-KRN7000 **5** in stimulating IL-2 from human NKT cells derived from hybridoma, while Chung et al.[37] observed a potent effect toward the secretion of IL-13 in other human iNKT cell clones (T_H2 bias). Altogether, these observations increase our knowledge on the sensitive and versatile role of CD1d and TCR receptors in the stabilization of GalCer substrates and highlight some sensitive differences between mice and human that remain difficult to rationalize.

CONCLUSION

In summary, after having shown that 4-deoxy-KRN7000 can be regarded as a full agonist of KRN7000 in the stimulation of human iNKT cells,[30] we have performed the synthesis of 3,4-dideoxy-3-fluoro **8** and 3,4-dideoxy-3,3-difluoro **9** KRN7000 analogues. Along with the aim to pursue our investigations on the ability of deoxy sphingosine derivatives to release selected cytokines from human and murine iNKT cells, substitutions of 3-OH group by one or two fluorine atoms on the ceramide substrate were sought to evaluate the importance of the TCR hydrogen-bond interactions through either a restored acceptor effect with Asp95 of the CDR3α-loop and/or an increasing donating NH-amide linkage with hTrp154 of the TCR.

In agreement with the literature, the loss of the hydroxyl H-donating group at C3 in the sphingoid base induced a destabilization of the GalCer/CD1d complex that could not be

balanced by its substitution with one isoelectronic fluorine atom. This result indicates a weak participation of donating Asp95-H···3-OH bond in the stabilization of the CD1d/Galcer/TCR ternary complex. Nevertheless, we found that addition of a second fluorine atom in 3,4-dideoxy-3,3-difluoro KRN7000 analogue **9** restores the stability of the ternary CD1d/GalCer/TCR complex both in vivo in mice and in vitro in human iNKT cells. Modeling studies showed that the *syn*-periplanar orientation of the 3(*R*)-F with NH amide group on the acyl chain enhances its donating effect with hTCR Trp154 stabilizing the ternary complex through the NH-bond.

Mice iNKT responses show strong divergences with human cells (already noted by others) and cannot be considered reliable to study partial agonists. In addition, the fact that no difference in IL-13 secretion was noted between all 3,4-dideoxy KRN7000 compounds **9**, **8**, and **5** when presented by Namalwa-CD1d, in contrast to HeLa-CD1d cells, suggests a distinct behavior depending on the antigen presenting cells.

Considering recent studies[56,57] showing that α-GalCer derivatives induce iNKT cell anergy, the discovery of novel immunostimulators able to shorten the unresponsiveness of human iNKT cells is of interest for therapeutic strategies. Thus, 3,4-dideoxy-α-GalCer analogues, which deliver a suitable cytokine secretion while maintaining T_H1 polarization, may pave the way toward candidates for anticancer immunotherapy.

EXPERIMENTAL SECTION

General. Solvents were purified and dried by standard methods prior to use.[58] ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300 (spectrometer fitted with a 5 mm i.d. BBO probe), Avance III 400 (spectrometer fitted with a 5 mm i.d. BBFO+ probe, temperature of the probe was set at 303K), and Avance III 500 (spectrometer fitted with a 5 mm i.d. ^{13}C/^{1}H dual cryoprobe, temperature of the probe was set at 303K). Spectra were recorded at room temperature with TMS as internal standard for ^{1}H spectra. Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz. All assignments were confirmed with the aid of two-dimensional ^{1}H, ^{1}H (COSYDFTP) or ^{1}H, ^{13}C (INVBTP) experiments using standard Bruker pulse programs. All reactions were monitored by TLC on commercially available precoated plates (Kieselgel 60 F_{254}), and the products were visualized with mostaine solution (250 mL of H_2O, 10.5 g of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.5 g of $Ce(SO_4)_2$, and 15 mL of H_2SO_4). Kieselgel 60, 230−400 mesh (Merck), was used for column chromatography. Optical rotations were measured at 20 ± 1 °C on Perkin-Elmer 341 in the indicated solutions whose concentrations are expressed in g/100 mL. Mass spectra were measured by CI with NH_3 on a quad. Hewlett-Packard 5989A. HRMS were measured on an LCT spectrometer from Micromass (Lokspray, channel 2795 from Waters, flow, MeOH/H_2O 50/50, 0.2 mL/min). FTIR spectra were obtained in the 500−4000 cm^{-1} range on a Bruker Vector 22 FT-IR spectrometer. The tested compounds gave >95% purity as determined by elemental analysis. Elemental analyses were performed at the Service Central d'Analyze—CNRS in Solaize (France).

(3R,4S)-4-(tert-Butyloxycarbonylamino)-3-fluoro-5-(2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)pent-1-ene ***16***. Under argon, DAST (262 μL, 1.99 mmol, 3.0 equiv) was added to a solution of alcohol **11** (490 mg, 0.66 mmol, 1.0 equiv) in CH_2Cl_2 (6.6 mL). The mixture was stirred for 10 min at rt and then quenched with saturated aqueous $NaHCO_3$ solution. The aqueous layer was extracted with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, and concentrated. Purification of the crude by flash chromatography on silica gel (EP/EtOAc 90:10 to 60:40) afforded fluoride **16** as a white solid (268 mg, 54%) and oxazolidinone **15** as a colorless oil (155 mg, 35%); $[\alpha]^{20}_D$ +39.0 (c 1.0, $CHCl_3$); mp 98−99 °C (crystallization: pentane/Et_2O 1:2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31−7.16 (m, 20H, H_{arom}), 5.84−5.70 (m, 1H, H_2), 5.19 (m, 3H, H_1, NH), 4.88 and 4.49 (AB syst, J = 11.4, 2H, CH_2Ph), 4.87 (md, J = 52.5, 1H, H_3), 4.80 and 4.57 (AB syst, J = 11.7, 2H, CH_2Ph), 4.79 (d, J = 3.6, 1H, $H_{1''}$), 4.71 and 4.69 (AB syst, J = 12.0, 2H, CH_2Ph), 4.40 and 4.30 (syst AB, J = 12.0, 2H, CH_2Ph), 3.98 (dd, J = 9.9, 3.6, 1H, $H_{2''}$), 3.87−3.80 (m, 4H, H_4, $H_{3''}$, $H_{4''}$, $H_{5''}$), 3.71 (m, 1H, $H_{6''}$), 3.65 (m, 1H, $H_{6''}$), 3.44 (m, 2H, H_5), 1.34 (s, 9H, O−C$(CH_3)_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 154.3 (CO), 137.7−136.8 ($C_q$ar), 132.4 (d, J = 18.8, C_2), 127.4−126.5 (CHar), 118.2 (C_1), 98.1 ($C_{1''}$), 90.6 (d, J = 172.5, C_3), 78.8 (O-$\underline{C}(CH_3)_3$), 78.6 ($C_{3''}$ or/and $C_{4''}$ or/and $C_{5''}$), 76.7 ($C_{2''}$), 74.8, 73.6, 73.4, 73.0 (CH_2Ph), 69.8 ($C_{3''}$ or $C_{4''}$ or $C_{5''}$), 69.1 (C_5), 67.8 ($C_{6''}$), 53.2 (d, J = 26.4, C_4), 28.4 (O−C($\underline{C}H_3)_3$). ^{19}F NMR (282 MHz, $CDCl_3$) δ −185.8 (dt, J = 52.5, 11.3). HRMS (ESI+) calcd for $C_{44}H_{52}FNNaO_8$ $[M + Na]^+$ 764.3575; found 764.3539. Anal. Calcd for $C_{44}H_{52}FNO_8$: C, 71.23; H, 7.06; F, 2.56; N, 1.89. Found: C, 71.21; H, 7.18; F, 2.79; N, 1.82.

(4S,5S)-(2,3,4,6-Tetra-O-benzyl-α-D-galactopyranosylmethyl)-5-vinyl-1,3-oxazolidin-(3H)-2-one ***15***. $[\alpha]^{20}_D$ +4.2 (c 1.3, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32−7.18 (m, 20H, Har), 6.20 (d, J = 9.6, 1H, NH), 5.76 (ddd, J = 17.1, 10.5, 6.6, 1H, $\underline{H}C{=}CH_2$), 5.28 (d, J = 16.8, 1H, HC=C$\underline{H}_2$), 5.18 (d, J = 10.5, 1H, HC=C$\underline{H}_2$), 4.86 and 4.57 (AB syst, J = 12.0, 2H, CH_2Ph), 4.78 and 4.67 (AB syst, J = 12.0, 2H, CH_2Ph), 4.71−4.69 (m, 2H, $H_{1''}$, CH_2Ph), 4.53−4.47 (m, 2H, H_5, CH_2Ph), 4.42 and 4.34 (AB syst, J = 11.7, 2H, CH_2Ph), 3.99 (dd, J = 10.5, 3.6, 1H, $H_{2''}$), 3.88−3.80 (m, 3H, $H_{3''}$, $H_{4''}$, $H_{5''}$), 3.65−3.59 (m, 2H, sugar−O-C$\underline{H}_2$, H_4) 3.48−3.35 (m, 3H, sugar−O-C$\underline{H}_2$, $H_{6''}$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.3 (CO), 138.4−137.6 ($C_q$ar), 134.0 ($\underline{H}C{=}CH_2$), 128.4−127.5 (CHar), 118.7 (HC=$\underline{C}H_2$), 98.6 ($C_{1''}$), 78.7 (C_5, $C_{3''}$ or $C_{4''}$ or $C_{5''}$), 76.4 ($C_{2''}$), 74.8 ($C_{3''}$ or $C_{4''}$ or $C_{5''}$), 74.6, 73.8, 73.5, 73.0 (CH_2Ph), 70.1 (sugar-O-$\underline{C}H_2$, $C_{3''}$ or $C_{4''}$ or $C_{5''}$), 69.5 ($C_{6''}$), 57.5 (C_4). HRMS (ESI+) calcd for $C_{40}H_{43}NaNO_8$ $[M + Na]^+$ 688.2886; found 688.2868

(2S,3R)-2-(tert-Butyloxycarbonylamino)-3-fluoro-1-(2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)heptadec-4-ene ***17***. To fluorine compound **16** (96 mg, 0.13 mmol, 1.0 equiv) dissolved in dry CH_2Cl_2 (2 mL) at room temperature under argon were added 1-tetradecene (330 μL, 1.30 mmol, 10.0 equiv) and Grubbs II catalyst (5 mg, 6 μmol, 0.05 equiv). The mixture was heated to reflux for 24 h. 1-Tetradecene (330 μL, 1.3 mmol, 10 equiv) and Grubbs II catalyst (5 mg, 6 μmol, 0.05 equiv) were added (after cooling) and the solution continued to stir for 4 h under reflux. Without treatment, CH_2Cl_2 was evaporated. Purification by flash chromatography on silica gel (petroleum ether/EtOAc 90:10) afforded **17** as a colorless oil (98 mg, 83%); $[\alpha]^{20}_D$ +36.6 (c 0.9, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41−7.26 (m, 20H, Har), 5.75−5.68 (m, 1H, H_5), 5.59−5.50 (m, 1H, H_4), 5.01 (d, J = 9.0, 1H, NH), 4.98 and 4.58 (AB syst, J = 11.2, 2H, CH_2Ph), 4.91 (d, J = 3.6, 1H, $H_{1''}$), 4.88 (m, 1H, H_3), 4.85 and 4.76 (AB syst, J = 12.0, 2H, CH_2Ph), 4.82 and 4.66 (AB syst, J = 11.6, 2H, CH_2Ph), 4.50 and 4.41 (AB syst, J = 12.0, 2H, CH_2Ph), 4.06 (dd, J = 10.0, 3.6, 1H, $H_{2''}$), 3.97−3.90 (m, 4H, H_2, $H_{3''}$, $H_{4''}$, $H_{5''}$), 3.83−3.73 (m, 2H, $H_{6''}$), 3.53 (d, J = 6.3, 2H, H_1), 2.02−1.99 (m, 2H, H_6), 1.43 (s, 9H, O−C$(CH_3)_3$), 1.35−1.27 (m, 20H, H_7−H_{16}), 0.90 (t, J = 6.6, 3H, H_{17}). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.2 (CO), 138.7−137.9 ($C_q$ar, C_5), 128.4−127.4 (CHar), 125.1 (d, J = 18.2, C_4), 99.1 ($C_{1''}$), 92.0 (d, J = 170.7, C_3), 79.4 (O-$\underline{C}(CH_3)_3$), 78.8 ($C_{3''}$ or $C_{4''}$ or $C_{5''}$), 76.7 ($C_{2''}$), 74.9 ($C_{3''}$ or $C_{4''}$ or $C_{5''}$), 74.4, 73.5, 73.1, 73.0 (CH_2Ph), 69.7 ($C_{3''}$ or $C_{4''}$ or $C_{5''}$), 69.0 (C_1), 68.1 ($C_{6''}$), 53.3 (d, J = 27.6, C_2), 32.3 (C_6), 31.9 (C_{15}), 29.6−28.8 (C_7−C_{14}), 28.3 (O−C($\underline{C}H_3)_3$), 22.6 (C_{16}), 14.1 (C_{17}). ^{19}F NMR (376 MHz, $CDCl_3$) δ −177.3 (d, J = 47.4). HRMS (ESI+) calcd for $C_{56}H_{76}FNNaO_8$ $[M + Na]^+$ 932.5453; found 932.5492.

(2S,3R)-3-Fluoro-2-(hexacosanoylamino)-1-(2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)heptadec-4-ene ***18***. The Boc protected amino compound **17** (108 mg, 119 μmol) in THF (1 mL) was treated with HCl solution (2 mol·L^{-1} in Et_2O, 580 μL, 10 equiv), and the mixture was heated to reflux until complete consumption of starting material. To the solution of the resulting amine dissolved in THF (2 mL) were added a 50% aqueous NaOAc solution (1 mL) and hexacosanoyl chloride (98 mg, 236 μmol, 1.9 equiv). The mixture was heated to 40 °C for 1 h 30 min and then diluted with THF and brine. The combined organic layer was washed with water combined, dried over $MgSO_4$. and concentrated. Purification of the residue by flash chromatography on

silica gel (EP/EtOAc 90:10) afforded *N*-acyl compound **18** as a white wax (54 mg, 38% over two steps); $[\alpha]^{20}_{D}$ +22.5 (*c* 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41−7.25 (m, 20H, Har), 6.18 (d, *J* = 9.0, 1H, NH), 5.77−5.69 (m, 1H, H_5), 5.55−5.47 (m, 1H, H_4), 4.94 and 4.58 (AB syst, *J* = 11.7, 2H, CH_2Ph), 4.98−4.86 (m, 1H, H_3), 4.89 (d, *J* = 3.6, 1H, $H_{1''}$), 4.82 and 4.76 (AB syst, *J* = 11.7, 2H, CH_2Ph), 4.81−4.66 (m, 2H, CH_2Ph), 4.49 and 4.40 (AB syst, *J* = 12.0, 2H, CH_2Ph), 4.22−4.17 (m, 1H, H_2), 4.06 (dd, *J* = 10.4, 3.6, 1H, $H_{2''}$), 3.98−3.89 (m, 4H, $H_{3''}$, $H_{4''}$, $H_{5''}$, H_1), 3.70−3.67 (m, 1H, H_1), 3.56−3.45 (m, 2H, $H_{6''}$), 2.38 (t, *J* = 7.4, 2H, $H_{2'}$), 2.04−1.99 (m, 2H, H_6), 1.58−1.53 (m, 2H, $H_{3'}$), 1.27−1.26 (m, 64H, H_7−H_{16}, H_4−$H_{25'}$), 0.89 (t, *J* = 6.9, 6H, H_{17}, $H_{26'}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8 ($C_{1'}$), 138.7−137.7 ($C_q$ar), 138.1 (d, *J* = 11.1, C_5), 128.4−127.2 (CHar), 125.2 (d, *J* = 18.3, C_4), 99.5 ($C_{1''}$), 91.9 (d, *J* = 170.3, C_3), 78.8 ($C_{3''}$), 77.2 ($C_{4''}$ or $C_{5''}$), 76.8 ($C_{2''}$), 74.8, 73.5, 72.9 (CH_2Ph), 70.0 ($C_{4''}$ or $C_{5''}$), 69.2 ($C_{6''}$), 68.1 (C_1), 51.9 (d, *J* = 27.5, C_2), 36.7 ($C_{2'}$), 32.3 (C_6), 31.9 (C_{15}, $C_{24'}$), 29.6−28.7 (C_7−C_{14}, C_4-$C_{23'}$), 25.6 ($C_{3'}$), 22.7 (C_{16}, $C_{25'}$), 14.1 (C_{17}, $C_{26'}$). ^{19}F NMR (376 MHz, $CDCl_3$) δ −178.9 (d, *J* = 47.7). HRMS (ESI+) calcd for $C_{77}H_{118}FNNaO_7$ $[M + Na]^+$ 1210.8790; found 1210.8789.

(2S,3R)-3-Fluoro-2-(hexacosanoylamino)-1-(2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl)heptadecane ***20*** *and (2S)-2-Hexacosanoylamino-1-(2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl)-heptadecane* ***19***. To derivative **18** (85 mg, 72 μmol, 1.0 equiv) dissolved in a $CHCl_3$/MeOH mixture (1:1, 3.7 mL) were added Pd/C 10% (85 mg) in one portion and AcOH (100 μL). The mixture was stirred overnight under H_2 atmosphere. The reaction mixture was diluted with pyridine, and the catalyst was removed by filtration over Celite. The filtrate was concentrated. The residue was diluted in a pyridine/Ac_2O mixture (2:1, 3 mL), and a catalytic amount of DMAP was added. The mixture was stirred overnight at rt. After evaporation of the solvent, the crude was purified by chromatography on silica gel (EP/EtOAc 90:10 to 80:20) to afford the fluorinated compound **20** (34 mg, 45%) and the 3-deoxy compound **19** (20 mg, 29%); 20 $[\alpha]^{20}_{D}$ + 33.9 (*c* 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.83 (d, *J* = 9.2, 1H, NH), 5.45 (d, *J* = 3.2,1H, $H_{4''}$), 5.33−5.29 (m, 1H, $H_{3''}$), 5.15−5.12 (m, 2H, $H_{1''}$, $H_{2''}$), 4.53 and 4.41 (2 m, 1H, H_3), 4.28−4.18 (m, 2H, H_2, $H_{5''}$), 4.16−4.02 (m, 2H, $H_{6''}$), 3.79−3.68 (m, 2H, H_1), 2.26−2.20 (m, 2H, $H_{2'}$), 2.14, 2.05, 2.04, 2.00 (4s, 12H, CH_3 Ac), 1.66−1.61 (m, 4H, H_4, $H_{3'}$), 1.33−1.25 (m, 68H, H_5−H_{16}, H_4-$H_{25'}$), 0.88 (t, *J* = 6.8, 6H, H_{17}, $H_{26'}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7 ($C_{1'}$), 170.3−170.1 (CO), 97.3 ($C_{1''}$), 92.8 (d, *J* = 173.0, C_3), 68.0, 67.9, 67.5 ($C_{2''}$, $C_{3''}$, $C_{4''}$, $C_{5''}$), 67.6 (C_1), 61.8 ($C_{6''}$), 51.2 (d, *J* = 23.0, C_2), 42.8 ($C_{2'}$), 32.1, 31.9, 29.6−29.3, 25.7, 22.7 (C_4−C_{16}, C_3−$C_{25'}$), 20.6 (CH_3 Ac), 14.1 (C_{17}, $C_{26'}$). ^{19}F NMR (376 MHz, $CDCl_3$) δ −191.1 (m). HRMS (ESI+) calcd for $C_{57}H_{104}FNNaO_{11}$ $[M + Na]^+$ 1020.7491; found 1020.7492. 19. $[\alpha]^{20}_{D}$ + 45.4 (c 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.51 (d, *J* = 8.8, 1H, NH), 5.45 (d, *J* = 3.2,1H, $H_{4''}$), 5.32 (dd, *J* = 3.2, 10.4, 1H, $H_{3''}$), 5.14−5.08 (m, 2H, $H_{1''}$, $H_{2''}$), 4.15 (m, 1H, $H_{5''}$), 4.10−4.02 (m, 3H, H_2, $H_{6''}$), 3.70 (dd, *J* = 10.0, 3.4, 1H, H_1), 3.49 (dd, *J* = 10.8, 3.4, 1H, H_1), 2.21−2.17 (m, 2H, $H_{2'}$), 2.14, 2.06, 2.04, 2.00 (4s, 12H, CH_3 Ac), 1.64−1.53 (m, 4H, H_3, $H_{3'}$), 1.36−1.18 (m, 70H, H_4−H_{16}, H_4−$H_{25'}$), 0.88 (t, *J* = 6.8, 6H, H_{17}, $H_{26'}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7 (CO), 170.4−170.1 (CO), 97.1 ($C_{1''}$), 71.0 (C_1), 68.2, 68.0, 67.6 ($C_{2''}$, $C_{3''}$, $C_{4''}$), 66.6 ($C_{5''}$), 61.9 ($C_{6''}$), 48.8 (C_2), 36.9 ($C_{2'}$), 31.9, 31.5, 29.7, 29.5, 29.4, 26.1, 25.8, 22.7 (C_3−C_{16}, C_3-$C_{25'}$), 20.7−20.6 (CH_3 Ac) 14.1 (C_{17}, $C_{26'}$). HRMS (ESI+) calcd for $C_{57}H_{105}NNaO_{11}$ $[M + Na]^+$ 1020.7491; found 1020.7491.

General Procedure for Deacetylation Step. To a stirred solution of the acetyl-protected glycolipid in a CH_2Cl_2/MeOH mixture (1:1) were added MeONa (1.2 equiv, solution in MeOH). After stirring at rt, resin H^+ Amberlyst 15 was added and the mixture was left to stir for additional 15 min. After filtration, the crude was subjected to column chromatography using silica gel (using the indicated eluent).

(2S,3R)-3-Fluoro-2-hexacosanoylamino-1-(α-D-galactopyranosyl)heptadecane ***8***. Unprotected glycoceramide **8** was synthesized following the general deacetylation procedure. From compound **20** (23 mg, 23 μmol) and MeONa (0.1 mM solution in MeOH, 400 μL, 46 μmol) in a CH_2Cl_2/MeOH mixture (2 mL), 3,4-dideoxy-3-fluoro-α-GalCer **8** (10 mg, 52%) was obtained as a white solid after purification by flash chromatography on silica gel ($CHCl_3$/MeOH 97:3); $[\alpha]^{20}_{D}$ + 26.4 (*c* 0.6, pyridine); mp 100−101 °C. ^{1}H NMR (500 MHz, pyridine-d_5) δ 8.66 (d, *J* = 9.0, 1H, NH), 5.42 (d, *J* = 3.8,1H, $H_{1''}$), 5.10 (m, J_{HF} = 50.0, 1H, H_3), 5.02−4.70 (m, 5H, OH, H_2), 4.66 (dd, *J* = 10.0, 3.8, 1H, $H_{2''}$), 4.57 (d, *J* = 2.9, 1H, $H_{4''}$), 4.42−4.44 (m, 3H, $H_{3''}$, $H_{5''}$, $H_{6''}$), 4.39 (dd, *J* = 10.8, 5.1, 1H, $H_{6''}$), 4.32 (ddd, *J* = 10.9, 3.7, 1.5, 1H, H_1), 4.19 (m, 1H, H_1), 2.48 (t, *J* = 7.5, 2H, $H_{2'}$), 1.95−1.82 (m, 4H, H_4, $H_{3'}$), 1.33−1.25 (m, 68H, H_5−H_{16}, H_4−$H_{25'}$), 0.88 (2t, *J* = 6.8, 6H, H_{17}, $H_{26'}$). ^{13}C NMR (125 MHz, pyridine-d_5) δ 173.4 (CO), 102.2 ($C_{1''}$), 93.9 (d, *J* = 172.0, C_3), 73.2, 71.6 ($C_{3''}$, $C_{5''}$), 71.4 ($C_{4''}$), 70.6 ($C_{2''}$), 68.2 (d, *J* = 4.1, C_1), 62.8 ($C_{6''}$), 52.8 (d, *J* = 24.0, C_2), 37.0 ($C_{2'}$), 32.1, 30.0−29.6, 26.3, 25.6 (C_4−C_{16}, C_3−$C_{25'}$), 14.3 (C_{17}, $C_{26'}$). ^{19}F NMR (376 MHz, pyridine-d_5) δ −188.73 (m). HRMS (ESI+) calcd for $C_{49}H_{96}FNNaO_7$ $[M + Na]^+$ 852.7069; found 852.7071. Anal. Calcd for ($C_{49}H_{96}FNO_7$·0.5$H_2$0): C, 70.12; H, 11.65; F, 2.26; N, 1.67. Found: C, 70.21; H, 11.88; F, 2.47; N, 1.53.

(2S)-2-Hexacosanoylamino-1-(α-D-galactopyranosyl)-heptadecane ***5***. Unprotected glycoceramide **5** was synthesized following the general deacetylation procedure. From **19** (18 mg, 18 μmol) and MeONa (0.4 mM solution in MeOH, 550 μL, 22 μmol) in CH_2Cl_2/MeOH (1 mL), 3,4-dideoxy-α-GalCer **5** (7 mg, 47%) was obtained as a white solid after purification by flash chromatography on silica gel ($CHCl_3$/MeOH 97:3); mp 91−92 °C. ^{1}H NMR (400 MHz, pyridine-d_5) δ 8.23 (d, *J* = 8.8, 1H, NH), 5.78−5.44 (m, 4H, OH), 5.40 (d, *J* = 3.6,1H, $H_{1''}$), 4.66 (dd, *J* = 10.0, 3.6, 1H, $H_{2''}$), 4.65 (m, 1H, H_2), 4.60 (m, 1H, $H_{4''}$), 4.54−4.49 (m, 2H, $H_{3''}$, $H_{5''}$), 4.49−4.39 (m, 2H, $H_{6''}$), 4.10 (dd, *J* = 10.2, 5.4, 1H, H_1), 3.91 (dd, *J* = 10.2, 5.4, 1H, H_1), 2.47 (t, *J* = 7.8, 2H, $H_{2'}$), 1.89−1.75 (m, 2H, $H_{3'}$), 1.42−1.26 (m, 72H, H_3−H_{16}, H_4−$H_{25'}$), 0.89−0.86 (m, 6H, H_{17}, $H_{26'}$). ^{13}C NMR (100 MHz, pyridine-d_5) δ 173.2 (CO), 101.7 ($C_{1''}$), 73.1 ($C_{3''}$ or $C_{4''}$ or $C_{5''}$), 71.8 (C_1), 73.1 ($C_{3''}$ and/or $C_{4''}$ and/or $C_{5''}$), 70.6 ($C_{2''}$), 62.9 ($C_{6''}$), 49.7 (C_2), 36.9 ($C_{2'}$), 32.3, 32.1, 30.0−29.6, 22.9 (C_3−C_{16}, C_4−$C_{25'}$), 26.6 ($C_{3'}$), 14.3 (C_{17}, $C_{26'}$). HRMS (ESI+) calcd for $C_{49}H_{97}NNaO_7$ $[M + Na]^+$ 834.7157; found 834.7184. Anal. Calcd for $C_{49}H_{97}NO_7$: C, 72.45; H, 12.04; N, 1.72. Found: C, 72.33; H, 12.20; N, 1.63.

Ethyl (2S)-2-(tert-Butoxycarbonylamino)-3,3-difluoro-hex-6-enoate ***26***. A mixture of the imine **24** (4.2 g, 9.3 mmol), prepared following Katagiri procedure,[48] and allyltri-*n*-butyltin (5.8 mL, 2 equiv) was dissolved in benzene (10 mL), and argon was bubbled through the solution for 15 min. The reaction mixture was then heated to 80 °C and stirred at this temperature for 5 min. AIBN (150 mg, 0.91 mmol 0.1equiv, in 5 mL benzene) was subsequently added in portions until complete consumption of the starting material (monitored by NMR, typically 4 h). The mixture was cooled to room temperature, diluted with ethyl acetate (50 mL), and a half saturated aqueous solution of KF (50 mL) was added. After 1 h of stirring, the biphasic system was filtered through Celite and the layers separated. The organic phase was dried over Na_2SO_4 and the solvent removed under reduced pressure. The oily residue was taken up in ethanol (20 mL), and 2 M aqueous HCl solution (20 mL) was added. The mixture was stirred at 50 °C until consumption of the imine (typically 1 h), and the mixture was then diluted with 20 mL of water. The aqueous phase was washed three times with diethyl ether. The solvent was removed under reduced pressure. The oily residue, corresponding to amine **25**, was taken up in dioxane (20 mL), and this solution was cooled to 0 °C (precipitation occurs). Boc_2O (1.5 eq relative to starting imine **24**) was added, followed by the dropwise addition of 50 mL of aqueous saturated $NaHCO_3$, and the mixture was stirred overnight. The reaction mixture was extracted with diethyl ether (3 × 50 mL), the combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure. The oily residue was purified by column chromatography (PE/AcOEt 4:1), affording the title compound **26** as an oil in 38% yield (from the imine). Data are consistent with the literature.[48]

(5S)-5-(tert-Butoxycarbonylamino)-4,4-difluoro-hex-1-en-6-ol ***22*** *and (5S)-5-(tert-butoxycarbonylamino)-4-fluoro-hex-1,3-dien-6-ol* ***27***. To the ester **26** (586 mg, 2 mmol), in 20 mL of absolute ethanol at 0 °C, was added $NaBH_4$ (380 mg, 10 mmol, 5.0 equiv) in one portion. After 10 min stirring, the ice bath was replaced by a water

bath and the solution was stirred for an additional 2 h, after which time TLC indicated that the reaction was complete. Saturated aqueous NH_4Cl was cautiously added (gas evolution) and the mixture stirred for 30 min. Then 100 mL of water and 50 mL of Et_2O were added, the layers were separated, and the aqueous phase was extracted with a further 50 mL of Et_2O. After drying over Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by silica gel chromatography (ethyl acetate) to afford the alcohol **22** in 80% yield and contaminated by 2−5% of the elimination product **27**. **22**: colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.75 (m, 1H, H_5), 5.25−5.05 (m, 3H, H_6, NH), 4.05−3.98 (m, 1H, H_2), 3.87−3.62 (m, 2H, H_1), 2.64 (td, J = 16.4, 7.0, 2H, H_4), 1.39 (s, 9H, *t*-Bu). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.8 (CO), 128.4 (C_5), 123.3 (t, J = 147.0, C_3), 80.4 ($C(CH_3)_3$), 60.2 (C_1), 54.6 (t, J = 23.4, C_2), 39.0 (t, J = 24.6, C_4), 28.2 (C_9). MS (EI): m/z = 252.1 (M + 1, 40%), 196.0 (M-^{t}Bu, 100%). **27**: Data for the elimination product **27** were extracted from a 1:1 mixture of the two compounds (obtained from another experiment where temperature was not strictly controlled). ^{1}H NMR (300 MHz $CDCl_3$) δ 6.51 (dt, J = 10.6, 17.2, 1H, H_5), 5.48 (dd, J = 10.8, 35.7, 1H, H_4), 5.15−5.00 (m, 3H, 2H_6, NH), 4.28 (m, H_2), 3.80−3.60 (m, 2H, H_1), 2.00 (br, 1H, OH), 1.38 (s, 9H, *t*-Bu). MS (EI) m/z = 232.1 (M + 1, 40%), 176.0 (M-^{t}Bu, 100%).

(5S)-5-(tert-Butyloxycarbonylamino)-4,4-difluoro-6-O-(2,3,4,6-tetra-O-benzyl-α-galactopyranosyl)-hex-1-ene ***21***. A Schlenk tube was charged with 4 g of activated 4 Å molecular sieves in powder, 1.2 g of $AgClO_4{\cdot}H_2O$ (5.7 mmol, 3 equiv) and 1.1 g (5.7 mmol, 3 equiv) of $SnCl_2$ and 15 mL of THF and the slurry mixture stirred at 0 °C under argon for 1 h. In a separate flask, the alcohol **22** (480 mg, 1.9 mmol) and galactosyl fluoride **12** (2 g, 2 eq, 3.8 mmol) were dissolved in diethyl ether (45 mL), and the solution was cooled to 0 °C. The second solution was added over 20 min to the Lewis acid, and the resulting mixture was stirred for 30 min at 0 °C and then 1 h at room temperature. The solution was quenched with aqueous saturated $NaHCO_3$, filtered over Celite, and the salts thoroughly washed with diethyl ether. The organic layer was dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude oily residue was taken up in THF, Boc_2O (1.5 equiv) and aqueous saturated $NaHCO_3$ were subsequently added, and the reaction mixture iwas stirred at rt overnight. The solution was diluted with 50 mL of water and extracted with 3 × 25 mL of diethyl ether. The organic layer was dried over Na_2SO_4 and the solvent removed under reduced pressure. Purification by column chromatography (PE/Et_2O 4:1) affords the title compound as an oil in 40% yield. Colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40−7.30 (m, 20H, Har), 5.71 (m, 1H, H_2), 5.17−4.98 (m, 3H, H_1 + NH), 4.86 and 4.48 (AB syst, J = 11.5, 2H, CH_2Ph), 4.77 (d, J = 3.6, 1H, $H_{1''}$), 4.73 and 4.57 (AB syst, J = 11.9, 2H, CH_2Ph), 4.71 and 4.63 (AB syst, J = 11.8, 2H, CH_2Ph), 4.42 and 4.32 (AB syst, J = 11.9, 2H, CH_2Ph), 4.07 (bs, 1H, H_5), 3.97 (dd, J = 10.0, 3.6, 1H, $H_{2''}$), 3.92−3.84 (m, 2H, $H_{4''}$, $H_{5''}$), 3.82 (dd, J = 10.8, 2.8, 1H, $H_{3''}$), 3.74 (dd, J = 10.8, 3.7, 1H, H_6), 3.65 (m, 1H, H_6), 3.44 (m, 2H, $H_{6''}$), 2.58 (td, J = 17.0, 7.2, 2H, H_3), 1.35 (s, 9H, *t*-Bu). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.4 (CO), 138.8−138.0 ($C_q$ar), 128.7−127.5 (CHar, C_2), 122.6 (t, J = 247.0, C_4), 120.1 (C_1), 99.0 ($C_{1''}$), 80.0 ($\underline{C}(CH_3)_3$), 78.8 ($C_{3''}$), 76.7 ($C_{2''}$), 75.1 ($C_{4''}$ or $C_{5''}$), 74.8 (CH_2Ph), 73.4 (CH_2Ph), 73.3 (CH_2Ph), 69.8 ($C_{4''}$ or $C_{5''}$), 69.0 ($C_{4''}$ or $C_{5''}$), 69.0 ($C_{6''}$), 66.3 (C_6), 53.6 (t, J = 26.0, C_5), 39.0 (t, J = 25.0, C_3), 28.3 ($C(\underline{C}H_3)_3$). ^{19}F NMR (376 MHz, $CDCl_3$) δ −105.4 (dd, J = 7.3, 232.0), − 106 (dq, J = 17.7, 232.0). HRMS (ESI+) Calc. for $C_{45}H_{53}F_2NNaO_8$ $[M + Na]^+$ 796.3631; found 796.3652.

(5S)-5-(Hexacosanoylamino)-4,4-difluoro-6-O-(2,3,4,6-tetra-O-benzyl-α-galactopyranosyl)-hex-1-ene ***29***. First, 300 mg (0.39 mmol) of (5*S*)-5-(*tert*-butyloxycarbonylamino)-4,4-difluoro-6-*O*-(2,3,4,6-tetra-*O*-benzyl-α-galactopyranosyl)-hex-1-ene **21** were dissolved in 25 mL of diethyl ether, and the solution was cooled to 0 °C. Subsequently, gaseous HCl was bubbled through the solution until complete disappearance of the starting material as judged by TLC. Excess HCl was removed by bubbling nitrogen through the solution, and additional Et_2O (10 mL) was added. Then, 2 equiv of $NaHCO_3$ (1.6 mL of a 0.5 M aqueous solution) were added at that temperature, and the mixture was stirred for a further hour at 0 °C. The organic layer was separated, and the aqueous layer extracted with another 10 mL of diethyl ether. After drying the organic layer over Na_2SO_4 and evaporating the solvent, the crude product was directly engaged in the next step. The crude amine **28** (260 mg) was dissolved in 10 mL of dry pyridine, *p*-nitrophenyl hexacosanoate (302 mg, 1.5 equiv/**21**, 0.504 mmol) was added in one portion, and the mixture was heated to 80 °C for 24 h. Pyridine was removed under vacuum, and the yellow solid residue was dissolved in diethyl ether. The solution was washed with 2 portions of a 0.5 M $NaHCO_3$ solution until the aqueous layer was colorless. The organic layer was dried over Na_2SO_4 and purified by column chromatography on silica gel using a 1:1 CH_2Cl_2/petroleum ether eluent to afford 160 mg of the title compound (approximately 38% yield) still contaminated with 5−10% of *p*-nitrophenol that could not be removed. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40−7.15 (m, 20H, Har), 6.33 (d, 1H, J = 9.1, NH), 5.75 (m, 1H, H_2), 5.87−5.13 (m, 2H, H_1), 4.85 and 449 (AB syst, J = 11.5, 2H, CH_2Ph), 4.77 (d, J = 3.9, 1H, $H_{1''}$), 4.75 and 4.57 (AB syst, J = 11.5, 2H, CH_2Ph), 4.72−4.65 (AB syst, J = 11.7, 2H, CH_2Ph), 4.43−4.31 (m + AB syst, 3H, CH_2Ph, H_5), 3.98 (dd, J = 10.0, 3.9, 1H, $H_{2''}$), 3.97−3.90 (m, 2H, $H_{6''}$, $H_{5''}$), 3.84−3.79 (m, 2H, $H_{4''}$, $H_{3''}$), 3.62 (dd, J = 11.7, 4.9, 1H, $H_{6''}$), 3.49 (dd, J = 10.0, 6.9, 1H, H_6), 3.36 (dd, J = 10.0, 5.9, 1H, H_6), 2.55 (td, J = 16.8, 7.0, 1H, H_4), 1.99−1.88 (m, 2H, $H_{2'}$), 1.45 (m, 2H, $H_{3'}$), 1.30−1.17 (m, 40H, H_4−$H_{23'}$), 0.80 (t, 3H, J = 7.0, $H_{24'}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4 ($C_{1'}$), 138.6−137.8 ($C_q$ar), 128.6−127.4 (CHar, C_2, C_3), 120.7 (C_1), 99.7 ($C_{1''}$), 78.8 ($C_{3''}$), 76.7 ($C_{2''}$), 74.9 ($C_{4''}$), 74.7 (CH_2Ph), 73.7 (CH_2Ph), 73.5 (CH_2Ph), 73.0 (CH_2Ph), 70.1 ($C_{5''}$), 69.5 ($C_{6''}$), 67.0 (C_1), 51.4 (m, C_5), 39.1 (m, C_3), 36.3 ($C_{2'}$), 31.9 ($C_{3'}$), 29.7−29.3, 25.4, 22.7 (CH_4-$C_{23'}$), 14.1 ($C_{24'}$). ^{19}F NMR (376 MHz, $CDCl_3$) δ −104.8 (d, J = 248.0), −105.8 (d, J = 248.0).

It should be noted that for compounds **29** and **30**, trace of a byproduct was observed on ^{19}F NMR spectra. This impurity could be assigned to a fluorinated ceramide residue resulting from hydrolysis of the glycosidic bound during HCl treatment for the Boc deprotection step. Trace of this side-product can be removed after the hydrogenation step.

(2S)-2-(Hexacosanoylamino)-3,3-difluoro-1-O-(2,3,4,6-tetra-O-benzyl-α-galactopyranosyl)-octadec-5-ene ***30***. Under an argon atmosphere, the amide **29** (54 mg) was dissolved in CH_2Cl_2 to obtain a 0.05 M solution, 5 equiv of 1-tetradecene (0.72 mmol, 180 μL) were added, and the mixture was heated to 40 °C. Once the temperature was stable, 5 mol % of Grubbs II catalyst (0.007 mmol, 6 mg) was added and the reaction stirred at that temperature until complete disappearance of the starting material (TLC monitoring, typically 3 h). After the reaction was complete, the mixture was cooled to room temperature and the product purified by column chromatography using a 1:1 CH_2Cl_2/PE eluent, affording the title compound **30** in 45% yield (28 mg). ^{1}H NMR (400 MHz) δ 7.40−7.15 (m, 20H, Har), 6.32 (d, J = 9.6, 1H, NH), 5.51 (dt, J = 6.5, 15.5, 1H, H6), 5.34 (dt, J = 6.5, 15.5, 1H, H5), 4.86 and 4.49 (AB syst, J = 11.4, 2H, CH_2Ph), 4.77, (d, J = 3.8, 1H, $H_{1''}$), 4.74 and 4.65 (AB syst, J = 11.8, 2H, CH_2Ph), 4.71 and 4.57 (AB syst, J = 11.8, 1H, CH_2Ph), 4.45−4.30 (m and AB syst, J = 11.8, 3H, CH_2Ph, H_2), 3.97 (dd, J = 10.1, 3.8, 1H, $H_{2''}$), 3.95−3.90 (m, 2H, $H_{5''}$, H_1), 3.85 (m, 1H, $H_{4''}$), 3.62 (dd, J = 11.8, 4.3, 1H, H_1), 3.49 (dd, J = 9.4, 6.7, 1H, $H_{6''}$), 3.36 (dd, J = 9.4, 6.0, 1H, $H_{6''}$), 2.48 (dt, J = 17.5, 6.7, 2H, H_4), 2.02−1.84 (m, 4H, $H_{2''}$, H_7), 1.60−1.45 (m, 2H, $H_{3'}$), 1.33−1.07 (m, 62H, H_4−$H_{23'}$ and H_8−H_{17}), 0.81 (t, J = 6.7, 6H, $H_{24'}$, H_{18}).). ^{19}F NMR (376 MHz, $CDCl_3$) δ = −104.9 (d, J = 247.0), δ = −105.7 (d, J = 247.0).

(2S)-1-O-(α-D-Galactopyranosyl)-2-hexacosanoylamino-3,3-difluoro-heptadecane ***9***. $Pd(OH)_2/C$ (20% w/w, 27 mg) was suspended in 1 mL of methanol, and H_2 was bubbled through the solution for 15 min. Then, the protected α-GalCer **32** (27 mg, 0.022 mmol), dissolved in 4 mL of chloroform, was added and the slurry was stirred under H_2 atmosphere. After 16 h, the solution was filtered through Celite and the cake washed 3 times with 10 mL of a warm $CHCl_3$/MeOH mixture (4:1). The solvent was removed and the crude purified by column chromatography on silica gel. Elution with $CHCl_3$/MeOH 98:2 then $CHCl_3$/MeOH 95:5 afforded 8 mg of the title compound **9** (40% yield) as a white solid. ^{1}H NMR (500 MHz, pyridine-d_5) δ 9.41 (d, J = 9.3, 1H, NH), 5.44 (d, J = 3.8, 1H, $H_{1''}$), 4.73 (dd, J = 9.9, 3.8, $H_{2''}$), 4.64 (bd, J = 3.0, 1H, $H_{4''}$), 4.59−4.51 (m, 3H, H_1, $H_{3''}$, $H_{5''}$), 4.51−4.42 (m, 2H, $H_{6''}$), 4.20 (dd, J = 10.9, 7.6,

1H, H_1), 2.63 (t, J = 6.9, $H_{2'}$), 2.37−2.17 (m, 2H, H_4), 1.99−1.82 (m, 2H, $H_{3'}$), 1.81−1.59 (m, 2H, H_5), 1.51−1.09 (m, 66H, $H_{4'\text{-}25'}$ and H_{6-17}), 0.91 (t, J = 7.2, 3H, $H_{26'}$ or H_{18}), 0.91 (t, J = 6.9, 3H, $H_{26'}$ or H_{18}). ^{13}C NMR (125 MHz, pyridine-d_5) δ 174.9 ($C_{1'}$), 125.3 (t, J = 246.0, C_3), 102.2 ($C_{1''}$), 73.4 ($C_{5''}$), 71.7 ($C_{3''}$), 71.1 ($C_{4''}$), 70.6 ($C_{2''}$), 66.8 (C_1), 62.8 ($C_{6''}$), 53.4 (t, J = 28.0, C_2), 37.2 ($C_{2'}$), 35.0 (t, J = 24.0, C_4), 32.3, 32.3, 30.2, 30.1, 30.0, 29.9, 29.9, 29.8, 29.8, 26.5 ($C_{3'}$), 23.1, 22.3 (C_5), 14.5. ^{19}F NMR (470 MHz, pyridine-d_5) δ −105.7 (d, J = 247.0), −106.4 (d, J = 247.0). HRMS (ESI+) calcd for $C_{50}H_{97}F_2NNaO_7$ $[M + Na]^+$ 884.7125; found 884.7144. Anal. Calcd for ($C_{50}H_{97}F_2NO_7 \cdot H_20$): C, 68.22; H, 11.34; F, 4.32; N, 1.59. Found: C, 68.49; H, 11.03; F, 4.16; N, 1.78.

In Vitro Assays for Human iNKT Cell Dtimulation. The ability of the 3,4-dideoxy-KRN7000 analogues **5**, **8**, and **9** to stimulate a human iNKT cell line was tested using human CD1d-expressing HeLa and Namalwa cells as presenting cells. iNKT cells were prepared from bulk human peripheral lymphocytes by two successive rounds of selection, using first an anti-Vα24 and, second, an anti-Vβ11 monoclonal antibody. At each round, cells were sorted using antimouse IgG-coated magnetic beads (Dynal) and cultivated in RPMI 1640 supplemented with 10% heat-unactivated fetal calf serum (FCS), 2 mM glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin (Gibco), and 300 U/mL IL-2 (Chiron). Human CD1d-transfected HeLa cells were obtained from M. Kronenberg (La Jolla, CA). Namalwa cells were transduced with a lentiviral vector containing a human CD1d insert in order to generate high expression of cell surface CD1d. These antigen presenting cells were cultivated in DMEM or RPMI 1640, respectively, containing 1 g/L glucose, supplemented as described above. Antigen presenting cells were plated at 30000 per well on 96-well flat bottom plates in complete RPMI for the TNF-α and IFN-γ secretion analyses and at 7500 per well for the IL-13 secretion analysis and incubated overnight at 37 °C with varying concentrations of glycolipids solubilized in DMSO. The cells were then washed twice with RPMI. Then 15000 iNKT cells in 150 μL of complete RPMI without IL-2 were then added for 6 h or 48 h at 37 °C for the TNF-α and IFN-γ or IL-13 analyses, respectively. Cell-free supernatants were collected and tested for the presence of either TNF-α by an MTT assay using WEHI 164 cells or IFN-γ and IL-13 by ELISA (BD Pharmingen). Experiments were repeated from 3 to 5 times for each glycolipid compound. Untransfected HeLa cells devoid of CD1d were used as negative control presenting cells. Synthetic KRN7000 **1** was used as reference in all experiments.

In Vivo Assay. Female mice C57BL/6J, 6−8 weeks old, purchased from Janvier laboratories, were bred and housed at INSERM, U892, University of Nantes, under the animal care license no. 44278. The stock solution of KRN7000 (1 mg/mL in DMSO) was dissolved with 10% DMSO in 0.9% NaCl solution (vehicle). Mice received intraperitoneally (ip) 100 μg/kg KRN7000 or GalCer analogues **5**, **8**, and **9** in 0.1 mL of vehicle. Groups of six mice were used for each time point.

ELISA. Sera were collected at different times points after each ip injection of glycolipid or vehicle. Serum IFN-γ, IL-4, and TNF-α levels were evaluated using specific ELISA kits (Mouse ELISA Ready-Set-GO, eBioscience) according to the manufacturer's instructions.

Statistical Analysis. To analyze the amounts of cytokines secreted at different time points following injection of glycolipids, areas under the curves (AUC) were compared. They were computed using the Prism 5.0 software (Graphpad, La Jolla, CA). Groups were then compared either by the Mann−Whitney 2-tailed test or by the Kruskal−Wallis test, and $p < 0.05$ values were considered as indicative of significant differences between groups.

■ ASSOCIATED CONTENT

Supporting Information

In vivo assay; in vivo secretion of cytokines in mice after ip injections of glycolipids; in vitro assay, RX data of **16**, theoretical model and NMR spectra of **5**, **8**, **9**, **16**−**20**, **21**, **22**, **27**, **29**, and **30**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; iNKT, invariant natural killer T; T_H, T helper; TCR, T cell receptor; CD, cluster of differentiation; hCD1d, human CD1d; mCD1d, mouse CD1d; SAR, structure−activity relationship; α-GalCer, α-galactosylceramide; rt, room temperature; DMAP, *N*,*N*-dimethylaminopyridine; Boc, *tert*-butyloxycarbonyl; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NMM, *N*-methylmorpholine; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*-dimethylformamide; AA, amino acids; Asp, aspartic acid; Arg, arginine; Thr, threonine; DMEM, Dulbecco's Modified Eagle's Medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; RPMI, Roswell Park Memorial Institute medium; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; PE, petroleum ether

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