

3,4-Dideoxy-3,3,4,4-tetrafluoro- and 4-OH epimeric 3-deoxy-3,3-difluoro-α-GalCer analogues: Synthesis and biological evaluation on human iNKT cells stimulation

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3,4-Dideoxy-3,3,4,4-tetrafluoro- and 4-OH Epimeric 3-Deoxy-3,3-difluoro-α-GalCer Analogues: Synthesis and Biological Evaluation on Human *i*NKT Cells Stimulation.

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Abstract

iNKT cells recognize CD1d/α-galactosylceramide (α-GalCer) complexes *via* their invariant TCR receptor and stimulate the immune response. Many α-GalCer analogues have been investigated to interrogate this interaction. Following our previous work related to the modification of the hydrogen bond network between α-GalCer and CD1d, we have now focused our attention on the synthesis of 3-deoxy-3,3-difluoro- and 3,4-dideoxy-3,3,4,4-

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tetrafluoro-α-GalCer analogues, and studied their ability to stimulate human *i*NKT cells. In each case, deoxygenation at the indicated positions was accompanied by difluoro introduction in order to evaluate the resulting electronic effect on the stability of the ternary CD1d/Galcer/TCR complex which has been rationalized by modeling study. With deoxy-difluorination at the 3-position, the two epimeric 4-OH analogues were investigated to establish their capacity to compensate for the lack of the hydrogen bond donating group at the 3-position. The 3,4-dideoxytetrafluoro analogue was of interest to highlight the amide *NH*-bond hydrogen bond properties.

Keywords

Fluoro GalCer analogues, iNKT activation, immune response, modeling study.

1. Introduction

CD1d restricted T lymphocytes, a subclass of lymphocytes, play a pivotal role in the innate-type immune response. A subpopulation of these CD1d restricted lymphocytes, called iNKT cells, feature a semi-invariant T receptor (TCR) that recognizes a variety of glycolipids antigens. In particular, recognition of glycosylceramides bound to CD1d protein receptors of antigen presenting cells (APCs) by iNKT-TCR leads to tertiary complex formation inducing expansion of their population and strong secretion of a large panel of T helper cytokines, including IFN-γ, TNF-α, and several interleukins.[1-9] These cytokines can stimulate the maturation of dendritic cells, activate the production of various cytokines, and stimulate other by-stander immune cells as cytotoxic CD8 lymphocytes. These mechanisms contribute to the inflammatory process, humoral immunity and antibody proliferation depending on two types of helper T cells polarization (T_H1 or T_H2). It was found that T_H1 cytokines (e.g. IFN-γ, IL-2) participate in cell-mediated immunity for tumor rejection and against infections,[10-15] while T_H2 cytokines (e.g. IL-4, IL-13) promote auto-immune responses, associated with a variety of diseases such as tuberculosis, type I diabetes, multiple sclerosis and rheumatoid arthritis.[16-22] 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.[23-25] Synthetic α-galactosylceramide α-GalCer **1** (also called KRN7000, Fig. 1)[26,27] has been considered as a promising agent against cancer[28-34] despite some undesired side effects as well as long-term NKT cell unresponsiveness following a first injection that restrict therapeutic development as a free drug in human.[35-38] Recent clinical trials have highlighted its

therapeutic potency as a potent adjuvant for vaccines[39-42] and in anticancer immunotherapy when preloaded on dendritic cells (DCs) or CD1d co-effector, or in combination with programmed cell death 1 (PD-1) blockade proteins.[43-48]

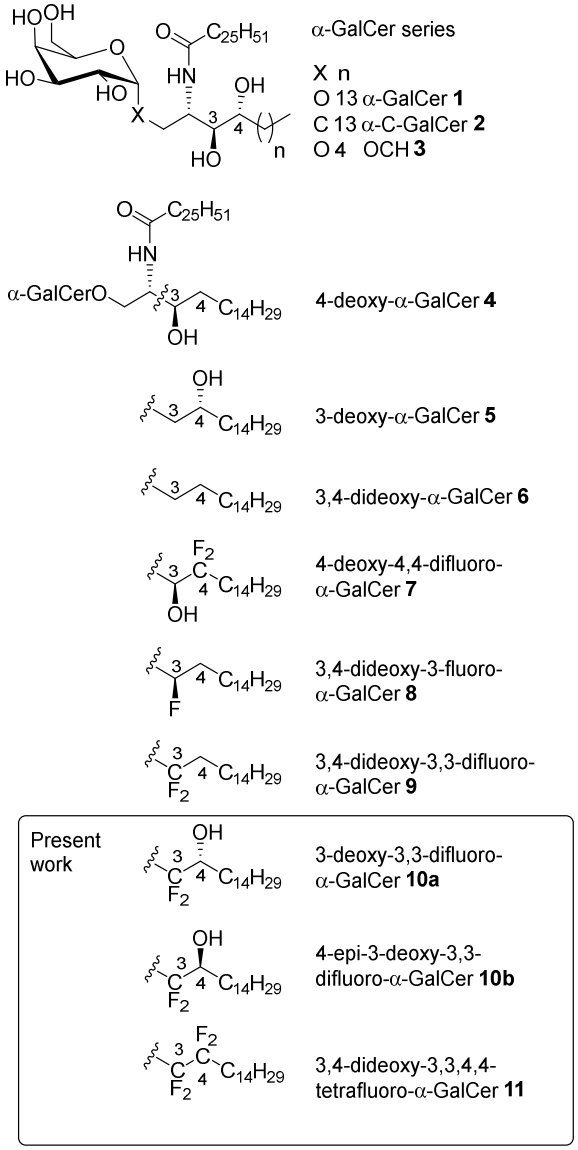


Fig 1. Structure of α-galactosylceramide analogues

The use of synthetic analogues of α-GalCer **1** targeting the T_H1/T_H2 balance has been extensively studied and well documented in excellent reviews.[23,49-52] Combinations of computational and crystal data,[53-57] with several structure-activity relationship studies on CD1d/α-GalCer analogues/TCR interactions established a relationship between stability of the ternary complex and T_H1/T_H2 polarisation of the immune response. After having shown the crucial importance of glycosidic α-configuration linkage,[58-60] replacement of the *O*-anomeric atom by a non-hydrolysable *C*-bond (α-*C*-GalCer **2**, Fig. 1) or ethylenic analogues

have produced potent derivatives for iNKT stimulation with T_H1 gain.[61-66] Unfortunately α-*C*-GalCer, appearing 1000-fold more potent than α-GalCer in mice, failed to satisfy clinical trials due to weak antigenic character on human iNKT cells. Other osidic linkages, *e.g.* thio[67-69] and amino analogues,[70,71] afforded versatile responses upon mouse or human iNKT cells without offering significant improvement in T_H1/T_H2 balance. Carbasugar[72-74] and open chains mimicking sugar architectures[75,76] have been shown to reinforce the T_H1 bias and to diminish the anergy phenomenon encountered with α-GalCer **1**.

Following indications of the relative influence of 2”-OH and 3”-OH hydroxyl groups on the sugar polar head interaction (2”-OH/Asp151-Cd1d and Gly96α -TCR; 3”-OH/Ser30-TCR) evidence was obtained for a preferential galactose configuration (4”-OH/Phe29α -TCR).[77-79] However, some recent data have emphasized a relative freedom for 6”-OH modifications of galactosyl moiety presenting *O*-methyl or acetyl group without alteration of the bioactivity. Furthermore, significant T_H1 bias has been observed with various 6”-deoxy derivatives bearing aromatic groups, such as phenyl, dansyl, biotinyl, pyridyl and naphthyl residues, introduced either through 6”-*N*-amido, carbamoyl, ureido and triazole linkages.[80-88] With a 6”-naphthyl ureido group, it has been suggested that the formation of an extra anchor *NH*-bond to the CD1d receptor results in a slight shift of the α-GalCer **1** ligand in the TCR grove leading to the T_H1-bias observed *in vivo*.[82,85] Recently, Van Calenberg *et al.*[89] have described a 6"-*O*-pyridinylcarbamoyl-α-*C*-GalCer analogue as potent iNKT agonist displaying high antigenic properties. Studies have been completed with other 6”-modified α-GalCer (6”-OMe, 6”-amidoalkyl and PEG chains…),[84,90] showing weak increase of iNKT stimulation without significant outcome on cytokine bias.

The modification of the ceramide fragment of the glycolipid is broadly accepted as a sensitive factor in terms of T_H1/T_H2 polarity. Derivatives in which the initial linear C_{26} acyl chain was replaced by unsaturated fatty acids,[91,92] branched[93] or amide containing[94] chain have been investigated and were suspected to use non-professional APC pathways to explain their T_H2 polarisation tendency.[95] Conversely, heterocyclic substitutions of shortened *N*-acyl chain derivatives mostly promote T_H1 orientation by installing adequate π-π stacking in the CD1d pocket[96,97] as suggested by aromatic-ended alkyl chains.[98-103]

Galactosylceramides featuring a truncated sphingosine chain mostly improve T_H2 response as illustrated by the well know sphingosine shortened OCH **3** derivative (Fig. 1) and its 4-deoxy analogues.[104-107] Indeed, the role of the two sphingosine hydroxyl groups at the C-3 and C-4 positions in the stability of the CD1d/α-GalCer/TCR complex was widely explored from

deoxy derivatives, 4-deoxy **4**, 3-deoxy **5** and 3,4-dideoxy **6** α-GalCer (Fig. 1)[78,103,104,108-112], and from epimeric[103,113] and polyhydroxylated,[114,115] amino[116] and amido[117] analogues. Although the role of the 4-OH group of α-GalCer in the interaction with human CD1d (Asp80-hCD1d) remains debated, the importance of the 3-OH interaction (Asp80-CD1d and Arg95 of the CDR3α-loop of the TCR) is fully established. These numerous efforts allowed to distinguish the effects of satellite hydrogen bonds on CD1d and TCR receptor interactions.

Then, introduction of fluorine atoms on the acyl or sphingosine chains of the ceramide appeared attractive to investigate modification of *H*-bonds in the interactions of GalCer ligands with CD1d *vs* TCR. Fluorination of bioactive compounds is often used to optimize properties.[118-122] While the blocking of metabolic sites is often achieved by fluorination, the strong fluorine electronegativity induces modification of a range of relevant properties, such as pK_a and hydrogen bond properties of adjacent functional groups, molecular conformation, and lipophilicity.[123-132] Tetrafluoroethylene (CF_2-CF_2) groups have received less attention as functional biological effectors,[133-137] relatively to –F and –CF_3 groups. CF_2 group has been shown to generate a widening of the C–CF_2–C angle (~111-118°) and a narrowing of the F–C–F angle (~100-104°) relative to tetrahedral geometry.[138,139]

Linclau *et al.*[140] have reported the synthesis of a 4-deoxy-4,4-difluoro-α-GalCer **7** analogue (Fig. 1) in which the *H*-bond donating capacity with CD1d was reinforced *vs.* a concomitant decrease in its ability to accept the *H*-bond from Asp-95 of the NKT TCR showing a weak loss on cytokine stimulation compared to α-GalCer **1** with a slight T_H1 bias. The latter outcome was completed by suppression of the 3-OH hydrogen-bond replaced by one or two fluorine atoms (3,4-dideoxy-3-fluoro-α-GalCer **8** and 3,4-dideoxy-3,3-difluoro-α-GalCer **9** respectively, Fig. 1).[112] It was observed that introduction of fluorine groups at 3-position of the sphinganine establishes a favourable *NH*-amide interaction from the acyl chain to the hTrp154 of the TCR resulting from the withdrawing electronic effect. This modification aims at partly compensate the lack of the 3-OH on the destabilisation of the CD1d/α-GalCer/TCR complex in human iNKT cells. The discrete role of *NH*-amide function, first suggested by Calenberg,[81-85,87,88] has been also highlighted by Linclau *et al.*[141] who found that amide neighbouring geminal (gem-) difluorine group, introduced at 2'-position of the acyl chain, leads to a T_H2 polarisation while iNKT stimulation level remains similar to α-GalCer **1**. This observation was confirmed by Hénon *et al.* in a molecular dynamic study[142] and T_H2 orientation of α-GalCer analogues presenting amide alteration have comforted this

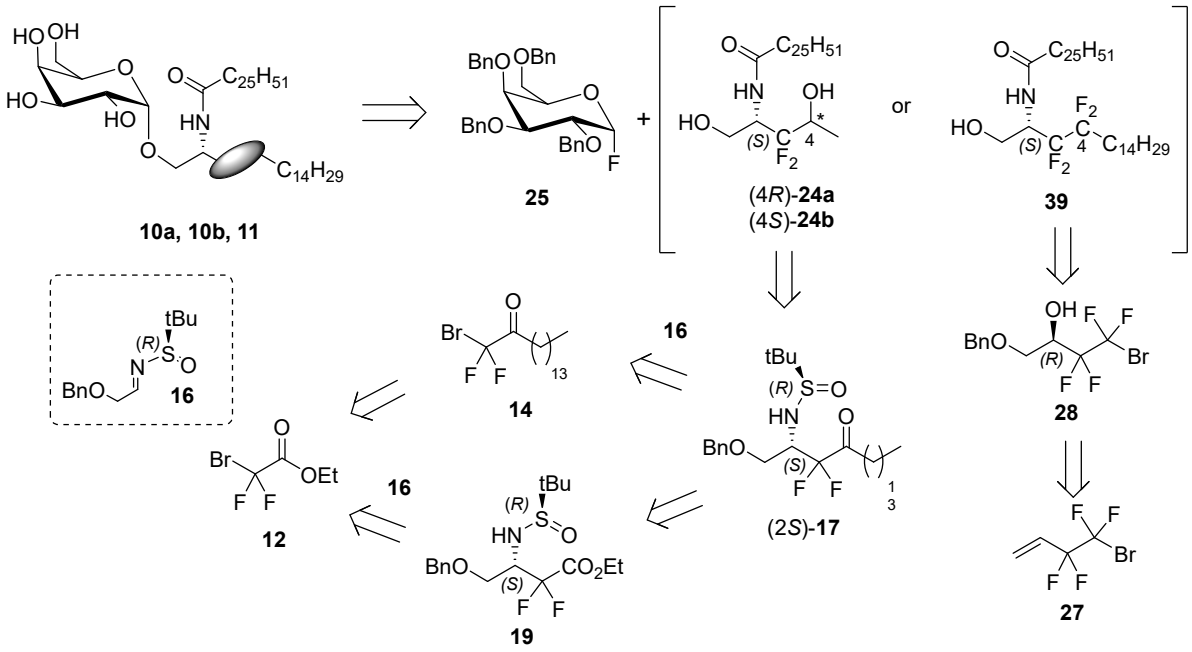
hypothesis, e.g., sulphonamide,[143] triazole,[84,87] ether and ester,[144] azetidine and pyrrolidine.[145]

The results obtained with fluorinated analogues of α-GalCer **1** inspired us to continue investigating this type of alteration on the CD1d/α-GalCer/TCR complex stability. In particular, the importance the 4-OH group will be evaluated through the two 3-deoxy-3,3-difluoro-α-GalCer analogue **10a** and its 4-OH epimer **10b**, in which the 3-OH is replaced by a *gem*- difluorine group. The 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11** analogue was also proposed as the next member of the 3,4-dideoxy analogues and to compare increasing fluorine's electronic effect with the 3,4-dideoxy-3,3-difluorinated **7** and 4-deoxy-4,4-difluoro **9** analogues previously evaluated (Fig. 1). Here we report both the synthesis of polyfluoro-α-GalCer analogues **10a**, **10b** and **11** and their *in vitro* biological evaluation on human iNKT stimulation.

2. Results and discussion

2.1. Chemistry

A conventional retrosynthetic analysis of the galactosylceramide targets **10** and **11** leads to a first disconnection between the galactose and the corresponding ceramide chains (Scheme 1), leading to the well-known fluoro galactosyl donor[146] and the two respective modified fluoroceramide analogues **24** and **39**. Functional group interconversion of the diastereomers **24a** and **24b** leads to the same ketone **17**. Further analysis leads to two distinct pathways, depending whether an imine addition disconnection is first executed, leading to **14** and the known chiral (*R*)-sulfinamine **16** or whether the alkyl chain is first disconnected, leading to the aminoester **19**.[147] Both intermediates **14** and **19** derive from the same bromodifluoroester **12**.

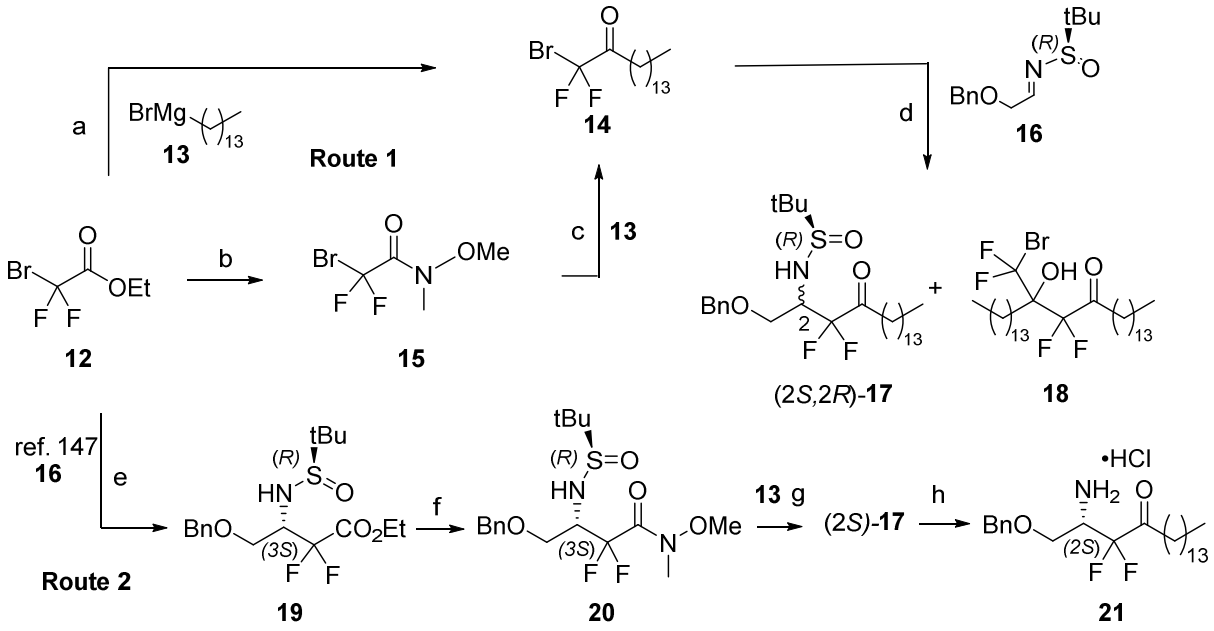


Scheme 1. Retrosynthetic pathway for analogues **10a**, **10b** and **11**.

From **39**, further retrosynthetic analysis relies on a series of functional group interconversions to introduce the amide bond and its amine precursor through dihydroxylation and alcohol to amine conversion. This proceeds *via* **28** to the commercially available fluorinated building block **27** by a known chemistry.[148]

2.1.1. Synthesis of 3-deoxy-3,3-difluoro-α-GalCer **10a** and its (4*S*)-OH epimer **10b**.

The synthesis of the sphingosine intermediate is shown in Scheme 2. The first approach involved introduction of the long alkyl chain by reaction of bromodifluoroester **12** with alkylmagnesiumbromide **13** to afford the bromoketone **14** (route 1).



Reagents and Conditions: (a) Et_2O, -78 °C, 19%; (b) MeNHOMe.HCl, $AlMe_3$, THF, r.t., 3 h, 62%; (c) THF, 25 °C, 30 min.; (d) $RhCl(PPh_3)_3$, Et_2Zn, THF, -20 to 0°C, 2 h, **17**: 27% [dr (2*S*):(2*R*), 82:18] and **18**: 36%; (e) $RhCl(PPh_3)_3$, Et_2Zn, THF, -20 to 0°C, 1 h, 43%; f) MeNHOMe.HCl, THF, *n*BuLi, -78°C for 4 h then -60 °C, 1 h, 92%; (g) THF, 0 °C, 40 min., then r.t., 1 h, 90%. h) 3M aq. HCl, 1,4-dioxane, r.t., 14 h, 95%

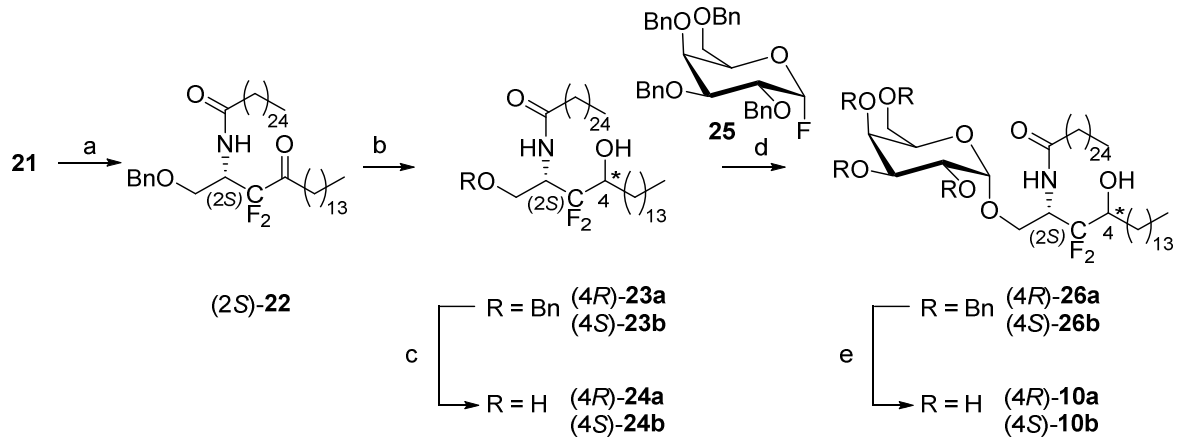
Scheme 2. Synthesis of key keto intermediate **21**

Hence, following the method of Kitazume *et al.,*[149] addition of ester **12** to Grignard reagent **13**, prepared *in situ* from tetradecanylbromide, afforded ketone **14** albeit in low yield (19%), while several side products prevailed. Consequently, addition of **13** to Weinreb amide intermediate **15**[150] was investigated. Unfortunately, while formation of the expected ketone **14** was observed in major amount, its isolation in pure form proved not possible. Nevertheless, Honda-Reformatsky reaction was attempted using the crude mixture with sulfinimine **16**[147] in the presence of $RhCl(PPh_3)_3$ and Et_2Zn. Following this route, the sulfinamine **17** was obtained in only 27% yield in an 82:18 3*S*/3*R* diastereomeric ratio, with the homocoupling product **18** being obtained as the major product (36%). Furthermore, attempts to separate the 2*S* and 2*R* isomers were unsuccessful.

An alternative route was thus investigated in which the introduction of the long alkyl chain would take place after installation of the chiral difluoroamine fragment (Scheme 2 - Route 2). Hence, Honda-Reformatsky reaction of difluoro ester **12** with (*R*)-sulfimine ester **16** yielded difluorinated sulfinamine ester (3*S*)-**19** isolated in 43% yield.[147] This time, conversion of the ester moiety to the corresponding Weinreb amide and chain extension proved successful: reaction of **19** with *N,O*-dimethylhydroxylamine hydrochloride mediated by *n*BuLi instead of trimethyl aluminium, afforded the Weinreb amide **20** in excellent yield, as was the subsequent

chain extension with Grignard reagent **13**. Removal of the sulfinyl group in (2*S*)-**17** using aq. HCl in dioxane[151] gave the amine **21** as hydrochloride salt in 95% yield. Acylation of **21** with cerotic acid was initially attempted under benzotriazol-1-yl-oxytripyrrolidinophosphonium (PyBOP) activation in dichloromethane (DCM) (Scheme 3). Despite a prolonged reaction time (40 h), the ceramide **22** was only produced in a modest 47% yield. An improved yield (73%) was obtained when carrying out the amide bond formation in refluxing DCM.

The two diastereomeric 3-deoxy-3,3-difluoride phytosphingosine analogues were then obtained by reduction of ketone **22** by $NaBH_4$, to give **23a** (52%) and **23b** (44%) which were separable by chromatography (Scheme 3). Establishment of the absolute alcohol configuration of **23a** and **23b** was achieved by ^1H NMR analysis of corresponding Mosher's ester derivatives (see Supporting Information SI1).



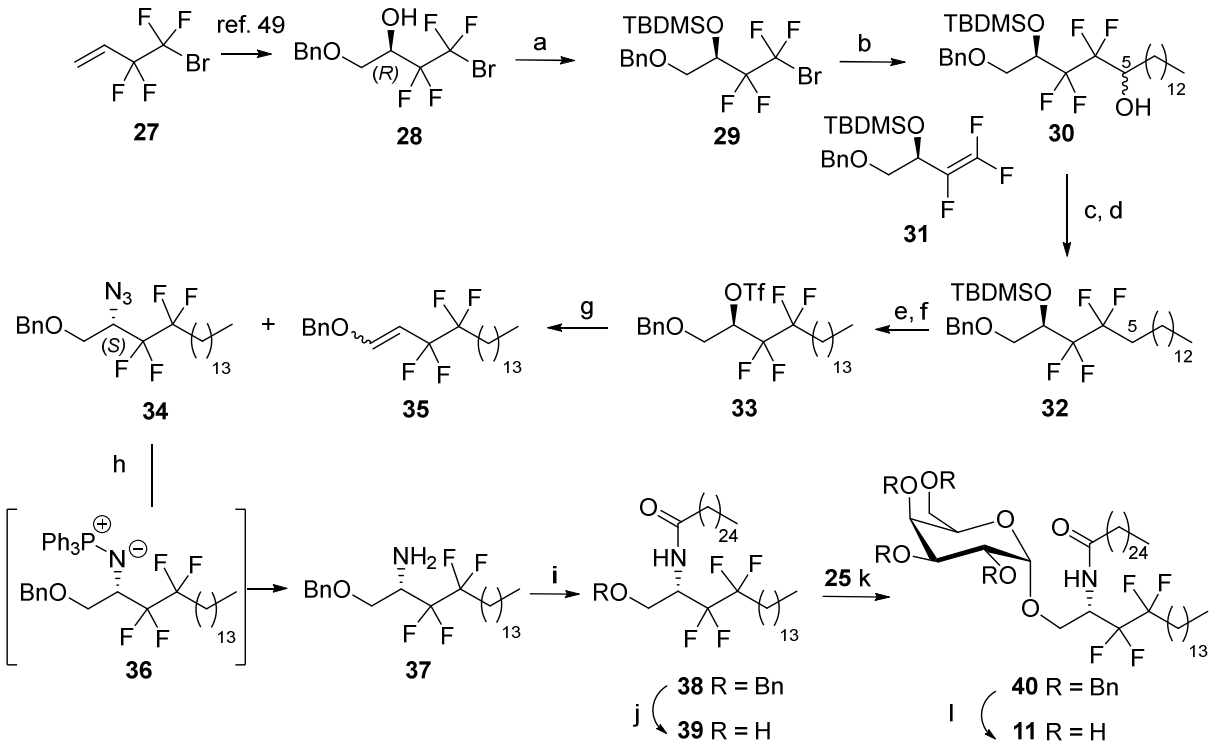
Reagents and Conditions: (a) Cerotic Acid, PyBOP, Et_3N, DCM, reflux, 20h, 73%; (b) $NaBH_4$, THF/EtOH (3:1), r.t., 2 h, **23a**: 52%, **23b**: 44%; (c) H_2, $Pd(OH)_2/C$, THF, r.t., 3 h, **24a**: 88% from **23a**, **24b**: 96% from **23b**; (d) $AgClO_4$, $SnCl_2$, 4Å MS, dark, THF, r.t., 2 h, **26a** from **24a**: 52%, **26b** from **24b**: 42%; (e) H_2, $Pd(OH)_2/C$, EtOH/$CHCl_3$ (4:1), r.t., 18 h, **10a**: 83% from **26a**, **10b**: 77% from **26b**.

Scheme 3. Synthesis of 3-deoxy-3,3-difluoro-α-GalCers **10a** and 4-OH epimer **10b**

Hydrogenolysis of the alcohols **23a** and **23b** cleanly delivered diols **24a** and **24b** in 88 and 96% yield, respectively. The alcohols **24** were then individually glycosylated with the perbenzylated galactosyl fluoride donor **25**. The resulting protected glycosides **26a** and **26b** were debenzylated yielding both 3-deoxy-3,3-difluoro-α-GalCer analogues **10a** and **10b** in 43% and 32% yield, respectively, over 2 steps.

2.1.2. Synthesis of 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11**

The synthesis of 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11** was performed in 12 reaction steps from the known enantiopure tetrafluoro (2*R*)-alcohol **28**[152] derived from alkene **27** *via* a stereoselective dihydroxylation and protection of the primary hydroxyl[148] (Scheme 4). It was decided to carry out the chain extension before the amine introduction. Hence, the alcohol group in **28** was protected using tert-butyldimethylsilyl chloride (TBDMSCl) in the presence of imidazole and *N*,*N*-dimethylaminopyridine (DMAP). Due to the deactivating effect of the halogenated appendix, reaction for 4 days at 50 °C was required to give **29** in 60% yield.



Reagents and Conditions: (a) TBDMSCl, Imidazole, DMAP, DMF, 50 °C, 4 d, 60%; (b) MeLi, tetradecanal, THF, -74 to -69 °C, 45 min, then to -55 to -50 °C, 1.5 h, **30**: 81% and **31**: 10%; (c) TCDI, DCE, r.t., 18 h, 95%; (d) AIBN, Bu_3SnH, Toluene, 110 °C, 40 min, 94%; (e) TBAF•$3H_2O$, THF, r.t., 40 min, 98%; (f) Tf_2O, pyridine, -40 °C for 1 h, then -40 to -10 °C for 1 h 30, 91%; (g) NaN_3, DMF, 0 °C, 1 h, then 50 °C, 14 h, **34**: 77% and **35**: 18%; (h) PPh_3, THF/H_2O, 60 °C for 13 h, 93%; (i) Cerotic acid, PyBOP, Et_3N, DCM, reflux, 21 h, 61%; (j) $Pd(OH)_2$/C, H_2, THF, r.t., 3 h, 94%; (k) **25**, $AgClO_4$, $SnCl_2$, 4Å MS, dark, THF, r.t., 2 h, **40**: 57%; (l) $Pd(OH)_2$/C, H_2, EtOH/$CHCl_3$ [4:1], r.t., 15 h, 85%.

Scheme 4. Synthesis of 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11**

Extension of the aliphatic chain was achieved through halogen-metal exchange followed by addition of tetradecanal. However, Li-halogen exchange from bromide **29** in the presence of long aliphatic aldehyde required an optimisation of the Konno's standard procedure[153,154] (Table 1).

Table 1. Optimization of conditions for the formation of alcohol **30** from **29**.

Entry	Method[a]	T (°C) *[t (min)]*	Yield (%)		
			29	**30**	**31**
1	A	-40 *[120]*	-	53	38
2	A	-78 *[120]*	61	21	-
3	B	-68 *[45]* to -40*[90]*	-	43	48
4	B	-78*[20]* to -50*[90]*	-	74	19
5	B	-78*[45]* to -50*[90]*	-	81	10

[a]Method A: MeLi (2.4 eq.) is added to a THF solution of **29** and tetradecanal; Method B: THF solution of **7** and tetradecanal is added to MeLi (2.4 eq.).

Adding MeLi to a solution of bromide **29** and tetradecanal in THF at -40°C (Method A) led to the desired alcohol **30** in 53 % yield (Table 1, entry 1). A byproduct **31** resulting from fluoride elimination was isolated in 38 % yield. Reducing the elimination event by working at lower temperature resulted in incomplete reaction due to precipitation of the aldehyde partner (Table 1, entry 2). Furthermore, the reaction was found to be very sensitive to the addition order of reagents and to the temperature (Table 1, entries 3-5). The best result was achieved by adding a solution of bromide **29** and tetradecanal in THF at -78°C to the solution of MeLi (Method B). After 45 min, the temperature was then increased up to -50°C and stirring was continued for another 90 min. This led to the formation of alcohols **30** as a 1/1 diastereomeric mixture in a reproducible 81% yield on 2 g scale along with a minor amount of alkene side product **31** (10 %). Barton-McCombie deoxygenation[155] of alcohols **30** underwent 5-deoxy intermediate **32** in 89% over 2 steps. Cleavage of the silylether with tetra-n-butylammonium fluoride (TBAF•3H_2O), followed by activation of (2*R*)-OH group as triflate led to **33** which was treated by NaN_3 in DMF to give the (2*S*)-azido derivative **34** in 68% yield over 3 steps. Interestingly, the formation of a mixture of alkene side products resulting from elimination process afforded a mixture of *E* and *Z*-tetrafluoro alkene **35** albeit in moderated yield (18%).

The reduction of azide **34** *via* the Staudinger reaction proved not straightforward. When the reaction was conducted 2 h at 25 °C, the iminophosphorane **36** was isolated as the sole product in 90 % yield. The stability of ylid **36** is likely due to a stabilization of the negative charge by the electron withdrawing effect of the tetrafluoroethylene group.[156-158] The addition of water to the reaction mixture (THF/H_2O [5:1]) did lead to the formation of the desired amine **37** in a low 30% yield, even after prolonged reaction time (14 h at 25 °C). However, by increasing temperature of the reaction to 60 °C for 13 h, complete hydrolysis of

the iminophosphorane **36** was performed and the target amine **37** was isolated as the sole product in 93% yield.

The ceramide glycosylation acceptor **39** was then obtained by combining amine **37** with hexacosanoic acid using PyBOP and coupling agent to afford **38** (61%) followed by hydrogenolysis of the primary benzyl ether (94% yield). Glycosylation with the perbenzylated galactosyl fluoride donor **25** using $AgClO_4/SnCl_2$ catalysis,[146] gave the protected α-galactoceramide **40** in 57% yield and final hydrogenolysis ($Pd(OH)_2/C$) in $EtOH/CHCl_3$ solution afforded the targeted 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11** in 85% yield.

2.2. Biological evaluation

The ability of the fluorinated GalCer analogues **10a**, **10b** and **11** to activate iNKT cells was then evaluated *in vitro* using Hela antigen-presenting cell lines transduced to express human CD1d (hHeLa-CD1d cells), and secretion of a T_H1 type cytokine IFN-γ, measured after a 6h stimulation, and a T_H2 type cytokine IL-13, measured after a 24h stimulation, was analyzed from human NKT cells (MAD11) prepared from bulk human peripheral lymphocytes (see Supporting Information SI2). hHeLa-CD1d cells of epithelial origin are inherently CD1d negative (result not shown) and IL-13 was chosen since IL-4 secretion was always extremely low in our *in vitro* assay conditions. Figure 2 shows the IFN-γ and IL-13 secretions induced by the iNKT MAD11 cell line after stimulation with antigen-presenting cells pulsed with canonical ligand α-GalCer (i.e KRN7000) and fluorine α-GalCer analogues **10a**, **10b** and **11**. A negative control confirms that when loaded to non-CD1d transduced HeLa cells the glycolipids induced only background quantities of IFN-γ and IL-13 release.

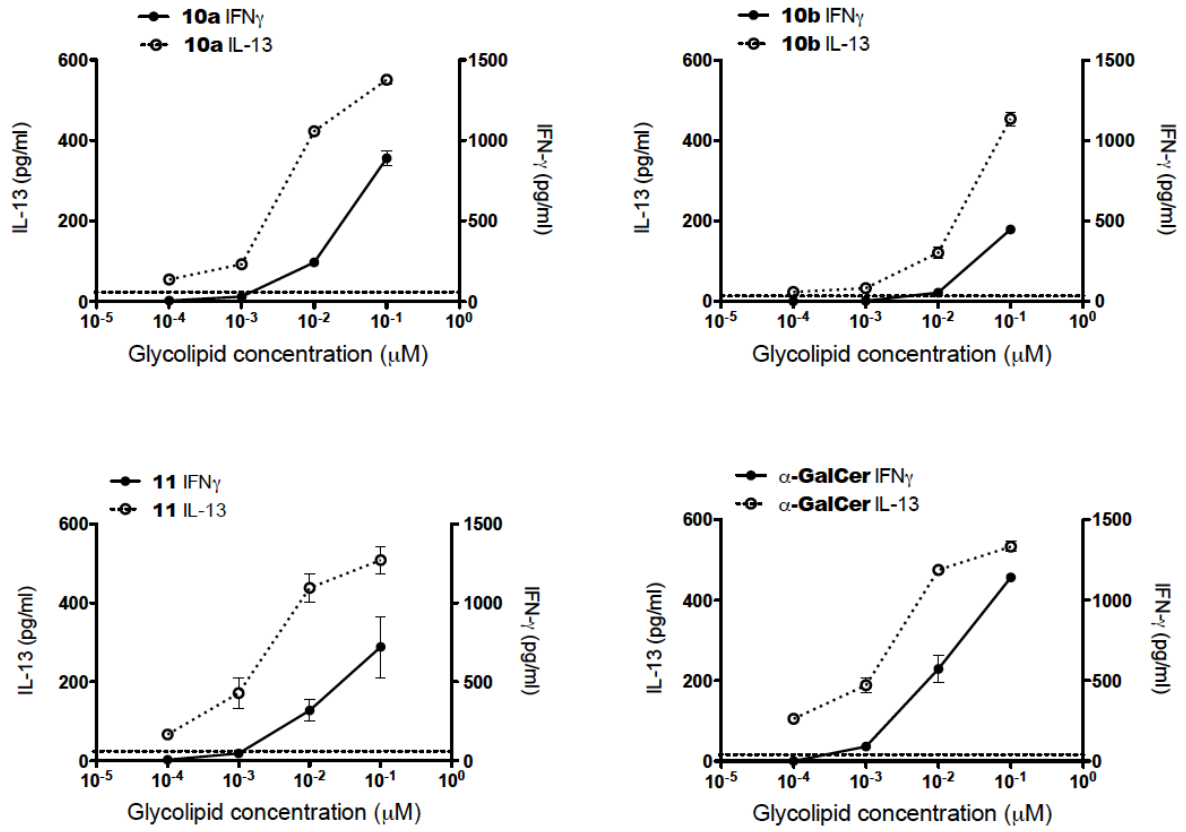


Figure 2. iNKT cell secretions of cytokines induced by fluorinated α-GalCer analogues **10a**, **10b** and **11**in hHeLa-CD1d cells of epithelial origin. Relative potencies of α-GalCer (KRN7000) and fluorinated compounds to stimulate IFN-γ (right y axis, solid lines), and IL-13 (left y axis, dashed lines) release by a human Vα24 iNKT cell line stimulated by CD1d-transfected Hela cells loaded with different concentrations of each glycolipid; a) top left: 3-deoxy-3,3-difluoro-α-GalCer **10a**, b) to right: 4-OH epimer **10b**, c) bottom left: 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11**, d) bottom right: **α-GalCer**. The mean release of cytokines into cell culture supernatants from triplicate wells were determined by ELISA and shown as pg/ml. Each graph is representative of at least two independent experiments. In absence of glycolipid or of CD1d, no IFN-γ secretion was detected and only very low IL-13 secretion was observed. The latter is shown by the horizontal dashed line.

Surprisingly, both the di and tetrafluorinated compounds **10a** and **11** induced secretions of cytokines at level similar to those induced by the reference α-GalCer **1**. These performances in the absence of 3-OH group can be ascribed to an increasingly favorable *NH*-amide interaction with the hTrp154 of the TCR due to electron withdrawing effects. However, the behaviour of gem-3-difluoro-4-OH series **10** and its analogues 3,4-dideoxy-3,3-difluoro-α-GalCer **9**, previously reported,[112] displaying the same and only a 20 fold lower agonist potency on hiNKT stimulation, respectively, question the real participation of the 4-OH group in a conventional H-bond with the hCD1d receptor. This questions remains intriguing, especially when 4-epi-analogue **10b**, with unnatural 4-OH configuration, expressed only 10-

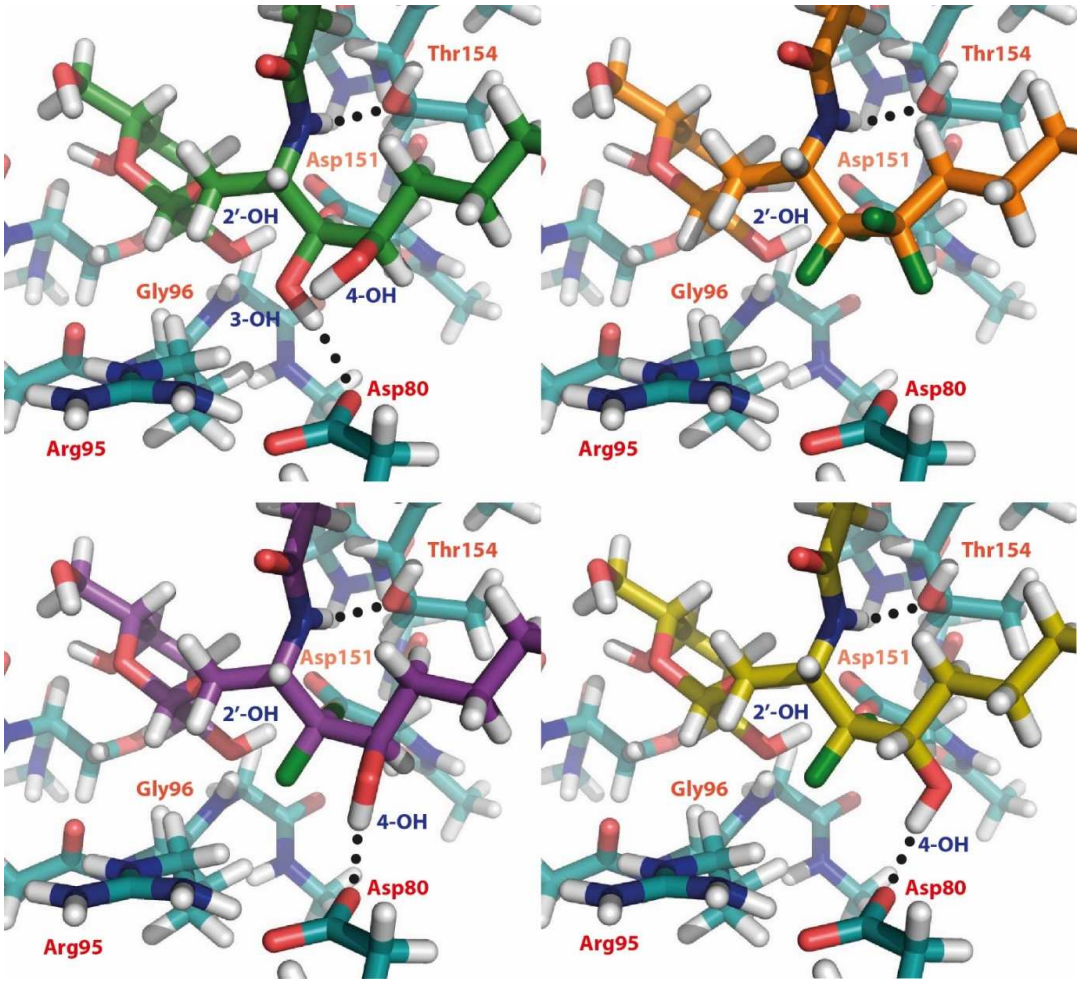
fold less potency than α-GalCer itself at inducing cytokines release. It is also noticeable that in all cases, and contrary to the reported poor agonist activity of monofluorinated 3,4-dideoxy-3-fluoro-α-GalCer **8** (Fig.1),[112] gem di- and tetrafluorinated groups introduced either at 3- or/and 4-positions are able to fully restore the ability of deoxy-GalCer derivatives to activate hiNKT cells.

2.3. Modelling study

The results obtained in human iNKT cells when presented by HeLa-CD1d transfected cells with fluorinated analogues, highlight a versatile individual contribution of 4-OH in the stability of the hCD1d/α-GalCer/TCR complex, even when accompanied by the withdrawing effect of vicinal 3,3-*gem*-difluoro group aimed at increase H-bond donating capacity. Beyond the loss of the hydrogen bond donor and acceptor capacity of the sphingosine OH groups, steric and/or conformational constraints induced by gem-difluoride group that could impair OH availability cannot be ruled out to explain such results. Last but not least, the result with 3,3,4,4-tetrafluorinated analogue **11**, showing no significant loss on the iNKT stimulation potency nor on the polarization of cytokines release, support the idea that a certain flexibility regarding OH groups is allowed on the ceramide while retaining iNKT activation.

Intrigued by these results, we sought for structural information that would shed light on the better understanding of the behavior of fluoro derivatives. A hybrid QM/QM' model (see the Supporting Information SI3) has been applied for **α-GalCer** (used as reference) and its fluorinated analogues, **10a**, **10b** and **11**, aiming to quantify their ability to interact with the surrounding amino acid residues. Seven amino acid residues, in direct interaction with the ligand in the CD1d/α-GalCer/TCR trimolecular complex crystal structure (PDB-ID 2PO6),[56] have been selected to design the model. The main optimized distances are reported in Table S1 (see SI3), whereas the energetic data are gathered in Table S2 (see SI3). In the optimized structure, the tetrafluorinated analogue **11**, lacking 3-OH and 4-OH hydroxyl groups, cannot exhibit hydrogen-bond interactions with Asp80, (Fig. 3) whereas in both analogues **10a** and **10b**, the 4-OH group establishes a shorter intermolecular interaction for which the distance appears to be dependant of the stereochemistry ($d_{(OH4...COO-)}$ = 2.025 Å in **10a** (4-*R*), and 1.808 Å in **10b** (4-*S*)) and shorter than in parent α-GalCer **1**. Nevertheless, it is worth noting that the interaction of Asp80 with **10a** and **10b** (ca. -15 kcal mol^{-1}) remains

lower than with α-GalCer **1** (-22.0 kcal mol^{-1}), in which 3-OH hydroxyl group takes advantage from an intramolecular H-bond activation from 4-OH ($d_{(OH4...OH3)}$ = 2.044 Å).[159] Conversely, a repulsive contribution prevails in the tetrafluorinated compound **11** (+3.5 kcal mol^{-1}). The presence of possible orthogonal multipolar C-F...C=O interactions as additional stabilizing interactions cannot be entirely ruled out. However, in the current optimized structures, the F...C distances, the C-F...C angles and the F...C=O angles (the criteria used to identify such interactions) are systematically outside the recommended ranges, indicating that such interactions cannot play a meaningful contribution. [126,160]



Legend: The designed QM/QM' model (PBE0/6-311G(d,p)/PBE0/6-31G) involves the Phe29, Ser30, Asp80, Arg95, Gly96, Asp151 and Thr154 residues. a) Top left: α-GalCer **1** in green. b) Top right: 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11** in orange. c) Bottom left: 3-deoxy-3,3-difluoro-α-GalCer **10a** in purple. d) Bottom right: 4-OH epimer **10b** in yellow. The dotted lines show the 3-OH…Asp80 in α-GalCer **1** and the 4-OH…Asp80 in **10a** and **10b**.

Figure 3. Comparison of the partially optimized structures of fluoro-α-GalCer derivatives within a model of the hCD1d/TCR binding site (Protein Data Bank code 2PO6)

In addition to its direct effect on the interaction with Asp80, we highlight that the ligand fluorination also tunes the interaction energies with the other amino acid residues. The most important pairwise interactions, ΔE, with the α-GalCer derivatives are observed with Asp151, from -43 kcal mol^{-1} (for α-GalCer **1**) to -35 kcal mol^{-1} (for **10a** and **10b**) and an intermediate value also being found (-39 kcal mol^{-1}) for the tetrafluorinated derivative **11**. An enhancement of ca. 3 kcal mol^{-1} of the interaction energies with the Arg95Gly96 residues is observed upon difluorination, from α-GalCer **1** to **10a**, **10b** and to **11** (Table S2 in the SI3). A closer examination reveals that the amide NH-bond of the ligands is interacting with the Thr154 hydroxyl group and despite the observed lengthening upon tetrafluorination, DFT indicates a slight increase of the pairwise interaction energy, ΔE (**11**, Thr154). Finally, the pairwise interaction energies with the Phe29Ser30 residues, which are interacting with the α-GalCer analogues through their carbohydrate moieties, are almost unaffected by fluorination, the ΔE values being almost unchanged (-15.6 *versus* -15.8 kcal mol^{-1}). In short, the computations show that the interaction energies of these seven amino acid residues with the ligands systematically decrease upon fluorination, the ΔE going from -90 kcal mol^{-1} with α-GalCer **1** to -80 kcal mol^{-1} with **10a** and **10b**, and -74 kcal mol^{-1} with **11**. With all the necessary precautions in interpreting the results of these simulations, it appears that the polyfluorinations in position 3 and 4 lead to a destabilization of the α-GalCer energies of interaction in the CD1d binding site.

Finally, it is worth noting that in their previous work, Baek *et al.* suggested, on the basis of molecular docking results, that the absence of the 3-OH hydroxyl group could be compensated by an interaction between the 4-OH group and the Tyr73 carbonyl group, accompanied by a lateral shift of the galactose headgroup toward the center of the binding groove.[110] Given Tyr73 is too far from the α-Galcer interacting sites, we initially did not include this residue in our model. We have therefore modified our model adding this eighth residue to investigate the interaction mode of analogue **10a** within the binding site defining a first starting geometry as found above and a second starting-point geometry as proposed by Baek *et al.* with the 4-OH···O=C(Tyr73) H-bond interaction ($d_{(OH4...O=C)}$ = 2.080 Å). Interestingly, it appears that after their geometry optimization the two final structures are very close, the galactose moiety of the second geometry shifting back to the first geometry, losing the 4-OH···O=C(Tyr73) H-bond interaction ($d_{(OH4...O=C)}$ = 3.297 Å). In this case, the 4-OH hydroxyl group does not interact with any residue, neither Asp80, nor Tyr73. Hence, it appears that the recovery of the 2'OH-COO$^-$(Asp151) and 3'OH-COO$^-$(Asp151), but also of

the NH···OH(Thr154) H-bond is prevailing over the 4-OH···O=C(Tyr73) H-bond interaction. The superposition of the two optimized structures is given in Figure S3 in SI3 (see supporting information). Finally, in absence of 3-OH on the sphingosine the computed interaction energies, ΔE = -86.4 and -85.6 kcal mol^{-1}, corroborate the presence of a stabilizing interaction between the 4-OH group and the Asp80, while, intramolecular H-bond between 4-OH and 3-OH could prevail in α-GalCer **1** to strengthen 3-OH…Asp80 interaction. This latter outcome is supported by a weak loss in cytokines release from iNKT previously observed by Linclau with the 4-deoxy-4,4-difluoro-α-GalCer derivative **7** (Fig. 1), accompanied by a slight T_H1 bias.[140] The expected increase in H-bond donating capacity of the 3-OH group due to the neighboring electron withdrawing gem-difluoro group at C4 could be attenuated by the loss of the intramolecular H-bond from the 4-OH group, explaining the weak biological improvement observed.

3. Conclusion

In conclusion, the synthesis of three novel α-GalCer analogues, 3-deoxy-3,3-difluoro-α-GalCer **10a** and its 4-OH epimer **10b** and 3,4-dideoxy-3,3,4,4-tetrafluoro-α-GalCer **11**, was achieved in ten and thirteen steps, respectively, to interrogate their molecular interactions at the atomic level with CD1d and TCR receptors. The results confirm the ability of the phytosphingosine fragment to adopt versatile conformational changes and to shift in the hCD1d binding groove to accommodate new interactions when lacking one or two OH structural ingredients. The only 10-fold lower potency of (4*S*)-OH epi-analogue **10b** to stimulate h*i*NKT compared to 3-deoxy-3,3-difluoro-α-GalCer **10a** supports this observation already mentioned by several authors from deoxy and diastereomeric analogues. Nevertheless, our study using 3-gem-difluorine derivatives seems to confirm that reinforcing the *NH*-amide donating capacity of 3-deoxy-phytosphinganine analogues tends to restabilize the CD1d/α-GalCer-analogue/TCR complex despite the loss of key contributing *H*-bonds on the phytosphingosine fragment. Obviously, the potency of 3,3,4,4-tetrafluoro-α-GalCer analogue **11** to stimulate h*i*NKT inducing IFN-γ and IL-13 secretions at the same level than α-GalCer **1**, pleads for this statement. However, observed similar T_H1/T_H2 bias suggests the lack of the *NH*-amide contribution on the polarisation of immune response. Although polarizing effects may be more efficiently observed in the *in vivo* mouse model setting,

however, this model suffers to not reflect properly human context due to higher level of available iNKT and can skew sensible information.

The observations made with fluorinated α-GalCer analogues may not only be due to a direct binding effect of the compounds to CD1d, but may also involve differences in uptake and subcellular localization owing to changes in hydrophobicity, especially upon polyfluorination. Nevertheless, our previous studies on deoxyfluoro sphingosine modified GalCer derivatives, supported by modeling along with the biological performances from these 3 new fluorinated analogues, point to an unidentified assistance of the 4-OH group on the key 3-OH contribution in the immune stimulation performance of α-GalCer (KRN7000), rather than a direct involvement through a proper H-bond with the CD1d receptor.

4. Experimental for Chemistry

Solvents were purified and dried by standard methods prior to use. Alternatively, the MB SPS-800-dry solvent system was used to dry dichloromethane and THF. Dry DMF solvent was commercially available from Sigma Aldrich and was used without purification. Glassware used for reaction was either flame dried under vacuum or under argon stream for several minutes. Reactions were carried out under rigorous anhydrous conditions and argon stream or positive pressure of argon. All reactions were monitored by TLC on commercially available precoated plates (Kieselgel 60 F254), and the compounds were visualized by UV (254 nm) when possible and with Ceric Ammonium Molybdate Solution [$(NH_4)_6Mo_7O_{24}$ (5g) + $Ce(SO_4)_2$ (0.2g) in H_2SO_4 5% solution (100 mL)] and heating. High purity grade (Merck grade 9385) pore size 60Å, 230-400 mesh particle size silica gel (Sigma Aldrich) was used for flash column chromatography. Solvents used for chromatography were prior distilled on a Buchi rotavapor R-220-SE. Melting points were determined on a RCH (C. Reichert) microscope equipped with a Kofler heating system. Optical rotations were measured at 20±1 °C with a Perkin–Elmer 341 instrument in the indicated solvents, and concentrations are expressed in g/100 mL. FTIR spectra were obtained in the 500–4000 cm^{-1} range with a Bruker Vector 22 FTIR spectrometer. ^{1}H , ^{13}C, ^{19}F and ^{31}P NMR spectra were recorded on a *Bruker Avance 300* spectrometer fitted with a 5 mm i.d. BBO probe carefully tuned to the recording frequency of 300.13 MHz (for ^{1}H), 75.47 MHz (for ^{13}C) and 282.40 MHz (for ^{19}F), the temperature of the probe was set at room temperature (around 293-294 K), on a *Bruker Avance 400* spectrometer fitted with a 5 mm i.d. BBFO+ probe carefully tuned to the recording frequency of 400.13 MHz (for ^{1}H), 100.61 MHz (for ^{13}C), 376.53 (for ^{19}F) and

121.49 MHz (for ^{31}P). The spectra are referenced to the solvent in which they were run (7.27 ppm for ^{1}H $CDCl_3$ [idem for $CDCl_3/CD_3OD$ 2:1] and 77.16 ppm for ^{13}C $CDCl_3$ [idem for $CDCl_3/CD_3OD$ 2:1], 3.58 and 1.73 ppm for ^{1}H THF-d_8 and 67.2 and 25.2 ppm for ^{13}C THF-d_8). Chemical shifts (δ are given in ppm, and coupling constants (J) are given in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or massif, br = broad and app = appeared as. All assignments were confirmed with the aid of two-dimensional ^{1}H, ^{1}H (COSY), or ^{1}H, ^{13}C (HSQC, HMBC) experiments using standard pulse programs. Low resolution mass spectrometry (MS) were recorded on a ThermoFinnigan DSQII quadripolar spectrometer (coupled with a TracUltra GC apparatus) for Chemical Ionization (CI) ; on a ThermoFinnigan LCQ Advantage spectrometer for ElectroSpray Ionisation (ESI).

Low and High resolution mass spectrometry (HRMS) were recorded on a ThermoFisher Scientific LTQ-Orbitrap spectrometer and on a Waters Xevo G2-XS Qtof spectrometer (coupled with an HPLC Acquity H-Class) for ESI ; on a Waters Xevo G2-XS Qtof spectrometer for ASAP+ ; on a Bruker Autoflex III spectrometer for MALDI+. Elemental analyses were performed with a Thermo Fisher Scientific Flash 2000 Series CHNS analyser, with detection by a catharometer (Thermal Conductivity Detector)

Synthesis of (2S,Rs)-1-Benzyloxy-3,3-difluoro-2-(tert-butyl)sulfinylaminooctadecan-4-one (2S)-17 via the route 1:

4.1. 1-bromo-1,1-difluorohexadecan-2-one **14**. Flame-dried Mg (82 mg, 3.36 mmol) was suspended in Et_2O (1 mL) and treated with a few drops of 1-bromotetradecane. The mixture was heated to reflux until effervescence was observed and then 1-bromotetradecane (1.00 mL, 3.36 mmol) was added over 10 min while heating and reflux was maintained an additional hour. After cooled at r.t., the resulting Grignard solution was added to a solution, at -78 °C, of ester **12** (393 µL, 3.06 mmol) in Et_2O (3 mL). The mixture was stirred at -78°C for 3 h, and then quenched with aq. HCl (3 M, 3 mL). The aqueous layer was extracted with Et_2O (3 × 3 mL) and the combined organic phases were dried ($MgSO_4$), filtered and concentrated to give a yellow oil. Column chromatography (petroleum ether /Et_2O 100:0 to 80:20) gave ketone **14** (202 mg, 19%) as colourless oil. *Data for **14**:* ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.78 (tt, J = 0.9, 7.3, 2H), 1.72–1.64 (m, 2H), 1.38-1.22 (m, 22H), 0.88 (t, J = 6.7, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.2 (*t*, J = 26), 114.3 (*t*, J = 319), 34.7, 32.1, 29.8–28.9 (9C), 23.1, 22.9, 14.2. ^{19}F NMR (376 MHz, $CDCl_3$) δ -64.7.

4.2. (2S,R$_S$*)-1-Benzyloxy-3,3-difluoro-2-(tert-butyl)sulfinylaminooctadecan-4-one* **(2*S*)-17** *and 17-(bromodifluoromethyl)-16,16-difluoro-17-hydroxyhentriacontan-15-one* **18**. To a solution of sulfinylimine (*R*$_S$,*E*)-**16**[147] (105 mg, 0.414 mmol) and $RhCl(PPh_3)_3$ (12 mg, 13 µmol) in THF (2.8 mL), at -20 °C, was added a solution of ketone **14** (176 mg, 0.495 mmol) in THF (0.5 mL) immediately followed by addition dropwise of Et_2Zn (1.0 M in hexane, 0.88 mL, 0.88 mmol). The mixture was warmed to 0 °C over 1 h, and then stirred for 1 h before being quenched with aq. NH_4Cl (sat., 3 mL). The aqueous layer was extracted with EtOAc (3 × 5 mL) and the combined organic phases were dried ($MgSO_4$), filtered and concentrated to give a brown oil. Column chromatography (petroleum ether /EtOAc/MeOH 90:10:0 to 0:90:10) gave compounds **18** (53 mg, 36%) as oily solid and (2*S*,2*R*)-**17** (59 mg, 27%) as a colourless oil.

4.2.1. *Data for* (2*S*)-**17***:* R_f 0.34 (petroleum ether/EtOAc 60:40). Mp 50–53 °C. $[\alpha]_D$ –17.5 (c 1.06, $CHCl_3$, 20 °C). IR (KBr) *ν* 3439, 3298, 2959, 2851, 1731, 1471, 1209, 1100, 1072 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) *δ* 7.38–7.24 (m, 5H), 4.55 (d, *J* = 11.7, 1H), 4.48 (d, *J* = 11.7, 1H), 4.06 (ddddd, *J* = 13.2, 11.4, 9.5, 5.7, 3.5, 1H), 3.89 (d, *J* = 9.4, 1H), 3.87 (dd, *J* = 9.5, 3.5, 1H), 3.74 (ddd, *J* = 10.0, 5.6, 1.4, 1H), 2.60 (br. t, *J* = 7.3, 2H), 1.51–1.47 (m, 2H), 1.33–1.19 (m, 22H), 1.22 (s, 9H), 0.89 (t, *J* = 7.1, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 200.4 (t, *J* = 29), 137.0, 128.3 (2C), 127.8, 127.7 (2C), 115.3 (t, *J* = 258), 73.5, 67.9, 57.8 (t, *J* = 25), 56.5, 37.5, 31.8, 29.7–28.7 (9C), 22.6, 22.3 (3C), 22.2, 14.0. ^{19}F NMR (376 MHz, $CDCl_3$) *δ* -113.3 (dd, *J* = 270, 12, 1F), -116.0 (dd, *J* = 270, 14, 1F). MS (CI+) m/z 530.3 $[M + H]^+$. HRMS (ESI+) for $C_{29}H_{49}F_2NO_3SNa^+$ $[M+Na]^+$ calcd. 552.3299, found 552.3285.

4.2.2. *Data for* **18***:* ^1H NMR (300 MHz, $CDCl_3$) *δ* 2.80-2.71 (m, 2H), 1.66-1.54 (m, 2H), 1.35–1.20 (m, 22H), 0.88 (t, *J* = 8.0, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 38.1, 32.1 (2C), 30.3-28.8 (28C), 22.8 (2C), 22.4, 14.3 (2C) [loss of 4C related to fluorine due to bad relaxation]. ^{19}F NMR (282 MHz, $CDCl_3$) *δ* -51.5 (ddd, *J* = 172, 10, 2, 1F), -52.8 (ddd, *J* = 172, 18, 4, 1F), -109.0 (d, *J* = 285, 1F), -115.6 (ddd, *J* = 285, 18, 10, 1F). MS (ASAP+) m/z 631.3 $[M + H]^+$. HRMS (ASAP+) for $C_{32}H_{60}F_4O_2Br^+$ $[M + H]^+$ calcd. 631.3713, found 631.3712.

4.3. *2-chloro-2,2-difluoro-N-methoxy-N-methylacetamide* **15**. To a suspension of *N,O*-dimethylhydroxylamine hydrochloride (2.28 g, 23.4 mmol) in THF (100 mL) at 0 °C was added dropwise $AlCl_3$ (1M in heptane, 23.4 mL, 23.4 mmol). After 40 min. at 0 °C, the

mixture was cooled to -40°C to add ethyl bromodifluoroacetate **12** (1 mL, 7.8 mmol) then warmed to r.t. and stirred for 3 h. Finally, the reaction was quenched at -40°C with aq. HCl (1.5 M, 100 mL). The aqueous layer was extracted with Et_2O (3 × 100 mL) and the combined organic phases were washed with brine (300 mL), dried ($MgSO_4$), filtered and concentrated to give an oil. Column chromatography (pentane/Et_2O 80:20) gave known compound **15**[161] (1.06 g, 62%) as a pale yellow oil.

Synthesis of (2S,R*S*)-1-Benzyloxy-3,3-difluoro-2-(tert-butyl)sulfinylaminooctadecan-4-one (2S)-17 via the route 2:

4.4. (3*S*,*R*S)-Ethyl 4-(Benzyloxy)-3-(*tert*-butylsulfinamino)-2,2-difluorobutanoate **19**. To a solution of sulfinylimine (*R*S,*E*)-**16**[147] (4.11 g, 16.20 mmol) and $RhCl(PPh_3)_3$ (450 mg, 0.49 mmol) in THF (120 mL), at -20 °C, was added bromoester **12** (6.23 mL, 48.60 mmol) and then dropwise Et_2Zn (1.0 M in hexane, 32.4 mL, 32.4 mmol). The mixture was warmed to 0 °C over 30 h and stirred for 1 h before being quenched with aq. NH_4Cl (sat., 90 mL). The aqueous layer was extracted with EtOAc (3 × 160 mL) and the combined organic phases were dried ($MgSO_4$), filtered and concentrated to give a brown oil. Column chromatography (petroleum ether /EtOAc/MeOH 90:10:0 to 0:90:10) gave sulfinylamide **19**[147] (2.67, 43%) as yellow oil.

4.5. *(3S,R*S*)-N-Methoxy-N-methyl-3-(tert-butyl)sulfinylamino-4-benzyloxy-2,2-difluorobutyr-amide* **20**. MeNHOMe·HCl (3.40 g, 34.9 mmol) was suspended in THF (70 mL) and the solution cooled to –78 °C prior adding, dropwise *n*BuLi (2.4M in hexane, 29 mL, 69.7 mmol). The mixture was stirred at –78 °C for 5 min then the cooling bath was removed for approximately 15 min. The reaction was then re-cooled to –78 °C and added with a solution of ester **19** (2.63 g, 6.97 mmol) in THF (85 mL). The reaction was stirred at –78 °C for 4 h then at –60 °C for 1 h before being quenched with aq. NH_4Cl (sat., 25 mL) and warmed to r.t., before addition of H_2O (50 mL). The aqueous layer was extracted with Et_2O (250 mL) and EtOAc (3 × 250 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated to give a light brown oil. Column chromatography (petroleum /EtOAc 70:30 to 50:50) gave the product **20** (2.52 g, 92%) as a pale yellow oil. R_f 0.19 (petroleum ether/EtOAc 50:50). $[\alpha]_D$ –29.7 (c 1.07, $CHCl_3$, 20 °C). IR (neat) *v* 3216, 2944, 2871, 1690, 1456, 1366, 1205, 1083 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.28 (m, 5H), 4.57 (d, *J* = 11.5, 1H), 4.53 (d, *J* = 11.7, 1H), 4.30 (m, 1H), 3.90–3.80 (m, 2H), 3.76 (dd, *J* = 10.1, 6.7,

1H), 3.72 (s, 3H), 3.11 (br. s, 3H), 1.23 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.4, 128.3 (2C), 127.9 (2C), 127.7, 115.8 (t, J = 256), 73.5, 68.1, 61.9, 58.5 (t, J = 24), 56.6, 33.0, 22.4 (3C)) [loss of 1C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, $CDCl_3$) δ -111.4 (dd, J = 262, 9, 1F), -112.7 (dd, J = 262, 14, 1F). MS (CI+) m/z 393.1 $[M + H]^+$. HRMS (ESI+) for $C_{17}H_{27}N_2O_4SF_2$ $[M+H]^+$ calcd. 393.1660, found 393.1668.

4.6. *(2S,R$_S$)-1-Benzyloxy-3,3-difluoro-2-(tertbutyl)sulfinylaminooctadecan-4-one* (2*S*)-**17**. Flame-dried Mg (305 mg, 12.5 mmol) was suspended in THF (74 mL) and treated with a few drops of 1-bromotetradecane. The mixture was heated to reflux until effervescence was observed then 1-bromotetradecane (3.69 mL, 12.4 mmol) was added over 60 min while heating. Reflux was continued for 1 h then the reaction was cooled to 0 °C and a solution of Weinreb amide **20** (974 mg, 2.48 mmol) in THF (15 mL) was added. Stirring was continued at 0 °C for 40 min then at r.t. for 1 h. The mixture was then cooled to 0°C before being quenched with aq. NH_4Cl (sat., 50 mL) then poured into H_2O (50 mL). The aqueous layer was extracted with EtOAc (3 × 100 mL) and combined organic layers were washed with brine (50 mL), dried ($MgSO_4$), filtered and concentrated to give an oil. Column chromatography (petroleum ether /EtOAc 100:0 to 0:100) gave Sulfinylamine (2*S*)-**17** as a colorless oil which became a white solid after storage (1.19 g, 90%). See data of **17** above.

4.7. *(2S)-2-Amino-1-benzyloxy-3,3-difluorooctadecan-4-one* **21**. Sulfunylamine (2*S*)-**17** (1.55 g, 2.93 mmol) was dissolved in 1,4-dioxane (35 mL) and treated with aq. HCl (3M, 9.8 mL, 29.3 mmol). The mixture was stirred at r.t. for 14 h then extracted with pentane (3 × 90 mL). A white solid was filtered from the pentane to give the product **21** (1.29 g, 95%) as the hydrochloride salt. R_f 0.59 (petroleum ether/EtOAc 10:90). Mp 111–115 °C. $[\alpha]_D$ +6.4 (c 1.0, $CHCl_3$, 20 °C). IR (KBr) ν 3432, 2918, 2850, 1744, 1123 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.09 (br. s, 2H), 7.36–7.24 (m, 5H), 4.54 (d, J = 11.5, 1H), 4.47 (d, J = 11.5, 1H), 4.27 (m, 1H), 3.97 (br. s, 2H), 2.58 (td, J = 6.9, 3.9, 2H), 1.41–1.37 (m, J = 6.9, 3H), 1.34–1.11 (m, 22H), 0.89 (t, J = 7.0, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.0, 136.5, 128.4 (2C), 128.0, 127.9 (2C), 113.4 (t, J = 258), 73.9, 64.7, 52.3 (dd, J = 26, 23), 37.0, 31.9, 30.3-28.4 (9C), 22.7, 22.1, 14.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -110.3 (dd, J = 280, 22, 1F), -116.3 (dq, J = 280, 14, 1F). MS (CI+) m/z 426.2 $[M + H]^+$. HRMS (ESI+) for $C_{25}H_{42}F_2NO_2^+$ $[M+H]^+$ calcd. 426.3184, found 426.3185.

4.8. *(2S)-1-Benzyloxy-3,3,difluorooctadecan-4-one-2-yl hexacosanamide* (2*S*)-**22**. Amine hydrochloride **21** (1.27 g, 2.74 mmol) was dissolved in $CHCl_3$ (146 mL) and cerotic acid (1.24 g, 3.12 mmol), PyBOP (1.62 g, 3.12 mmol) and Et_3N (0.83 mL, 5.94 mmol) were added to the solution. The mixture was stirred at reflux for 20 h then diluted with DCM (130 mL). The organic phase was washed with H_2O (130 mL) and brine (130 mL), dried ($MgSO_4$), filtered and concentrated to give an off-white solid, which was suspended in MeOH and filtered. The resultant white solid was purified by column chromatography (petroleum ether/DCM 50:50 then 0:100) to give amide (2*S*)-**22** (1.60 g, 73%) as a white solid. R_f 0.50 (petroleum ether/EtOAc 80:20). Mp 93–97 °C. $[\alpha]_D$ +15.5 (c 0.96, $CHCl_3$, 20 °C). IR (KBr) *v* 3311, 2917, 2849, 1736, 1657, 1546 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.23 (m, 5H), 6.12 (d, *J* = 9.3, 1H), 4.83 (dddd, *J* = 16.6, 12.0, 9.0, 4.2, 1H), 4.47 (s, 2H), 3.73 (dd, *J* = 10.2, 3.9, 1H), 3.58 (dd, *J* = 10.2, 4.7, 1H), 2.62 (dt, *J* = 7.3, 6.5, 2H), 2.21 (t, *J* = 7.6, 2H), 1.55–1.40 (m, 6H), 1.37–1.17 (m, 64H), 0.89 (t, *J* = 6.7, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 137.0, 128.5 (2C), 128.0, 127.7 (2C), 73.5, 67.1, 50.9 (t, *J* = 26), 37.3, 36.6, 31.9, 30.2–28.6 (31C), 25.5, 22.7, 22.4, 14.1 (2C) [loss of 2C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, $CDCl_3$) δ -113.1 (dd, *J* = 266, 12, 1F), -115.5 (dd, *J* = 266, 12, 1F). MS (CI+) m/z 805.0 $[M + H]^+$. HRMS (MALDI+) for $C_{51}H_{92}F_2NO_3^+$ $[M + H]^+$ calcd. 804.7040, found 804.7026.

4.9. *(2S)-1-Benzyloxy-3,3-difluorooctadecan-4-ol-2-yl hexacosanamide* **23**. Amide **22** (1.02 g, 1.26 mmol) was dissolved in THF/EtOH (3:1, 60 mL) and $NaBH_4$ (72 mg, 1.90 mmol) was added to the solution. The mixture was stirred at r.t. for 2.5 h then quenched with H_2O (1.1 mL) and stirring was continued for 20 min before the solution was concentrated under reduced pressure to give a white solid. Column chromatography (petroleum ether/EtOAc 95:5, 90:10) gave (4*R*)-**23a** (531 mg, 52%) and (4*S*)-**23b** (448 mg, 44%) both as white solids.

4.9.1. *Data for isomer (4R)-**23a**:* R_f 0.26 (petroleum ether/EtOAc 80:20). Mp 85–89 °C. $[\alpha]_D$ +15.7 (c 0.7, $CHCl_3$, 20 °C). IR (KBr) *v* 3432, 3304, 2917, 2850, 1654, 1551, 1464, 1100 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.44–7.28 (m, 5H), 5.93 (d, *J* = 8.9, 1H), 4.85–4.68 (m, 1H), 4.58 (d, *J* = 11.8, 1H), 4.54 (d, *J* = 11.8, 1H), 3.84–3.67 (m, 2H), 3.63 (ddd, *J* = 10.2, 6.1, 1.0, 1H), 3.09 (d, *J* = 6.8, 1H), 2.22 (t, *J* = 7.6, 2H), 1.86–1.41 (m, 6H), 1.15–1.39 (m, 66H), 0.89 (t, *J* = 7.0, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.5, 137.0, 128.6 (2C), 128.1, 127.9 (2C), 122.0 (dd, *J* = 254, 249), 73.5, 71.8 (dd, *J* = 30, 26), 67.4, 50.1 (t, *J* = 25), 36.7, 31.9, 30.0–28.9 (32C), 25.7, 25.6, 22.7, 14.1 (2C). ^{19}F NMR (376 MHz, $CDCl_3$) δ -114.9

(ddd, J = 254, 15, 7, 1F), -119.5 (dt, J = 254, 14, 1F). MS (CI+) m/z 807.0 $[M + H]^+$. HRMS (ESI+) for $C_{51}H_{94}F_2NO_3$ $[M + H]^+$ calcd. 806.7202, found 806.7221.

4.9.2. *Data for isomer (4S)-**23b**:* R_f 0.53 (petroleum ether/EtOAc 80:20). Mp 88–90 °C. $[\alpha]_D$ –2.3 (c 0.8, $CHCl_3$, 20 °C). IR (KBr) ν 3330, 2918, 2849, 1655, 1539, 1470, 1100 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.43–7.29 (m, 5H), 6.11 (d, J = 8.3, 1H), 4.74 (d, J = 4.5, 1H), 4.56 (s, 2H), 4.60 (ddt, J = 25.9, 8.5, 4.6, 1H), 3.91 (dd, J = 10.5, 4.7, 1H), 3.75 (ddd, J = 10.5, 4.2, 2.0, 1H), 3.52 (ddd, J = 23.6, 8.3, 4.0, 1H), 2.26 (td, J = 7.5, 2.2, 2H), 1.70–1.55 (m, 6H), 1.35–1.21 (m, 66H), 0.89 (t, J = 7.0, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.5, 137.4, 128.5 (2C), 128.0, 127.7 (2C), 121.9 (dd, J = 255, 252), 73.4, 69.2 (dd, J = 32, 24), 65.6, 49.2 (dd, J = 33, 23), 36.5, 31.9, 30.0–29.0 (31C), 27.4 (d, J = 4), 25.9, 25.6, 22.7, 14.1 (2C). ^{19}F NMR (376 MHz, $CDCl_3$) δ -121.8 (dd, J = 253, 25, 1F), -124.7 (dd, J = 253, 24, 1F). MS (CI+) m/z 807.0 $[M + H]^+$. HRMS (MALDI+) for $C_{51}H_{93}F_2NO_3Na^+$ $[M + Na]^+$ calcd. 828.7016, found 828.6985.

4.10. *(2S,4R)-3,3-Difluorooctadecan-1,4-diol-2-yl hexacosanamide* (4*R*)-**24a**. Benzyl protected ceramide (4*R*)-**23a** (477 mg, 0.592 mmol) was dissolved in THF (8.3 mL) and treated with $Pd(OH)_2/C$ (20%, 125 mg, 0.178 mmol). The reaction mixture was flushed with H_2 then stirred under H_2 atmosphere for 3 h before being filtered through Celite®. The pad was rinsed with warm THF. Concentration of the filtrate gave a white solid, which was purified by column chromatography (DCM/MeOH 99:1 to 90:10) to give ceramide (4*R*)-**24a** (372 mg, 88%) as a white solid. R_f 0.08 (petroleum ether/EtOAc 70:30). Mp 95–101 °C. $[\alpha]_D$ +7.8 (c 0.5, THF, 20 °C). IR (KBr) ν 3422, 3339, 2919, 2850, 1652, 1545, 1473, 1070 cm^{-1}. ^1H NMR (400 MHz, THF-d_8) δ 7.10 (d, J = 9.3, 1H), 4.66–4.50 (m, 1H), 4.47 (d, J = 7.8, 1H), 4.12 (t, J = 6.1, 1H), 3.77–3.59 (m, 3H), 2.16 (t, J = 7.4, 2H), 1.69–1.40 (m, 6H), 1.38–1.21 (m, 66H), 0.89 (t, J = 7.1, 6H). ^{13}C NMR (100 MHz, THF-d_8) δ 173.4, 123.9 (dd, J = 252, 249), 72.1 (dd, J = 29, 26), 61.1 (t, J = 4), 53.3 (t, J = 24), 36.8, 33.0, 30.9–30.3 (32C), 27.0, 26.7, 23.7, 14.6 (2C). ^{19}F NMR (376 MHz, THF-d_8) δ -119.4 (ddd, J = 253, 16, 8, 1F), -121.4 (ddd, J = 253, 16, 12, 1F). MS (CI+) m/z 717.0 $[M + H]^+$. HRMS (MALDI+) for $C_{44}H_{87}F_2NO_3Na^+$ $[M + Na]^+$ calcd. 738.6548, found 736.6532.

4.11. *(2S,4S)-3,3-Difluorooctadecan-1,4-diol-2-yl hexacosanamide* (4*S*)-**24b**. Benzyl protected ceramide *(4S)-**23b*** (418 mg, 0.518 mmol) was dissolved in THF (8.3 mL) and treated with $Pd(OH)_2/C$ (20%, 125 mg, 0.178 mmol). The reaction mixture was flushed with

H_2 then stirred under H_2 atmosphere for 3 h before being filtered through Celite®. The pad was rinsed with warm THF. Concentration of the filtrate gave a white solid, which was purified by column chromatography (DCM/MeOH 99:1, 96:4) to give ceramide (4*S*)-**24b** (358 mg, 96%) as a white solid. R_f 0.1 (petroleum ether/EtOAc 70:30). Mp 100–105 °C. $[\alpha]_D$ –10.4 (c 0.87, $CHCl_3$, 20 °C). IR (KBr) *v* 3343, 3303, 2915, 2850, 1623, 1472 cm^{-1}. ^1H NMR (400 MHz, THF-d_8) δ 7.56 (d, *J* = 8.3, 1H), 5.10 (d, *J* = 4.3, 1H), 4.37 (app. dtd, *J* = 26.1, 8.3, 3.6, 1H), 3.90 (t, *J* = 5.3, 1H), 3.89–3.83 (m, 1H), 3.73–3.62 (m, 1H), 3.54–3.43 (m, 1H), 2.26 (t, *J* = 7.3, 2H), 1.69–1.43 (m, 6H), 1.39–1.21 (m, 66H), 0.89 (t, *J* = 6.9, 6H). ^{13}C NMR (100 MHz, THF-d_8) δ 176.5, 70.0 (dd, *J* = 32, 24), 59.0, 53.4 (dd, *J* = 32, 23), 36.4, 32.9, 31.1–30.5 (30C), 30.3 (t, *J* = 8), 28.5 (d, *J* = 4), 27.0, 26.6, 23.6, 14.5 (2C). ^{19}F NMR (376 MHz, THF-d_8) δ -123.6 (dd, *J* = 249, 26, 1F), -126.3 (dd, *J* = 249, 24, 1F) [loss of 1C related to fluorine due to bad relaxation]. MS (CI+) m/z 717.1 $[M + H]^+$. HRMS (ESI+) for $C_{44}H_{87}F_2NO_3Na^+$ $[M + Na]^+$ calcd. 738.6548, found 736.6531.

4.12. *1-O-(2,3,4,6-Tetra-O-benzyl-α-galactosyl)-(2S,4R)-3,3-difluorooctadecan-1,4-diol-2-yl hexacosanamide* (4*R*)-**26a**. In the dark, $SnCl_2$ (230 mg, 1.21 mmol), $AgClO_4$ (251 mg, 1.21 mmol) and ground 4Å molecular sieves (1.66 g) were combined in THF (2.8 mL) and stirred at r.t. for 90 min. In parallel, ceramide (4*R*)-***24a*** (289 mg, 0.404 mmol) was dissolved in THF (3.8 mL) and added to a solution of fluoro galactosyl donor **25**[162] (329 mg, 0.606 mmol) dissolved in THF (5.3 mL). Then, the mixture containing Lewis acids was added with (4*R*)-***24a*** and **25**, *via* cannula, to the mixture of Lewis acids beforehand cooled to 0°C and stirring was maintained, in the dark, for 20 min. The mixture was warmed to r.t., stirred for 2 h and then filtered through Celite®. The mixture was stirred at r.t. in the dark for 2 h, then filtered through Celite®. The pad was rinsed with EtOAc (~110 mL) and the filtrate was washed with aq. $NaHCO_3$ (sat., 5 × 14 mL), dried ($MgSO_4$), filtered and concentrated to give a residue. Column chromatography (petroleum ether/EtOAc 90:10 to 80:20) gave (4*R*)-**26a** (260 mg, 52%) as a white solid. R_f 0.50 (petroleum ether/EtOAc 70:30). Mp 85–86 °C. $[\alpha]_D$ +28.6 (c 1.0, $CHCl_3$, 20 °C). IR (KBr) *v* 3629, 3317, 2919, 2850, 1652, 1617 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.51–7.09 (m, 20H), 6.07 (d, *J* = 8.7, 1H), 4.92 (d, *J* = 11.4, 1H), 4.87 (d, *J* = 3.9, 1H), 4.84 (d, *J* = 12.5, 1H), 4.80 (d, *J* = 11.8, 1H), 4.72 (d, *J* = 11.7, 2H), 4.82–4.68 (m, 1H), 4.56 (d, *J* = 11.5, 1H), 4.47 (d, *J* = 11.7, 1H), 4.39 (d, *J* = 11.8, 1H), 4.14 (d, *J* = 6.8, 1H), 4.06 (dd, *J* = 10.1, 3.6, 1H), 3.94 (d, *J* = 1.8, 1H), 3.91 (t, *J* = 6.5, 1H), 3.82 (dd, *J* = 10.1, 2.6, 1H), 3.87–3.74 (m, 2H), 3.68 (dd, *J* = 11.7, 8.0, 1H), 3.55–3.46 (m, 2H), 2.13 (td, *J*

= 7.8, 2.3, 2H), 1.77–1.47 (m, 5H), 1.45–1.06 (m, 67H), 0.90 (t, *J* = 6.8, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1, 138.3, 138.3, 137.7, 137.5, 128.6–127.1 (20C), 122.4 (dd, *J* = 255, 248), 99.0, 79.0, 75.7, 74.6, 74.6, 73.8, 73.4, 73.0, 70.8 (dd, *J* = 31, 25), 70.0, 68.9, 66.1, 50.3 (t, *J* = 24 Hz), 36.4, 31.8, 29.8–29.0 (31C), 28.7, 25.9, 25.4, 22.6, 14.0 (2C). ^{19}F NMR (376 MHz, $CDCl_3$) δ -117.4 (dd, *J* = 251, 17, 1F), -120.1 (ddd, *J* = 251, 21, 7, 1F). MS (MALDI+) m/z 1260.9 $[M + Na]^+$. HRMS (ESI+) for $C_{78}H_{121}F_2NO_8Na^+$ $[M + Na]^+$ calcd. 1260.8952, found 1260.8998.

4.13. *1-O-(2,3,4,6-Tetra-O-benzyl-α-galactosyl)-(2S,4S)-3,3-difluorooctadecan-1,4-diol-2-yl hexacosanamide* (4*S*)-**26b**. In the dark, $SnCl_2$ (199 mg, 1.05 mmol), $AgClO_4$ (218 mg, 1.05 mmol) and ground 4Å molecular sieves (1.47 g) were combined in THF (2.4 mL) and stirred at r.t. for 90 min. In parallel, ceramide (4*S*)-**24b** (250 mg, 0.349 mmol) was dissolved in THF (3.8 mL) and added to a solution of fluoro galactosyl donor **25**[162] (284 mg, 0.531 mmol) dissolved in THF (4.5 mL). Then, the solution containing **25** and (4*S*)-**24b** was added, *via* cannula, to the mixture of Lewis acids beforehand cooled to 0°C and stirring was maintained, in the dark, for 20 min. The mixture was warmed to r.t., stirred for 2 h and then filtered through Celite®. The pad was rinsed with EtOAc (~100 mL) and the filtrate was washed with aq. $NaHCO_3$ (sat., 5 × 12 mL), dried ($MgSO_4$), filtered and concentrated to give an off-white residue. Column chromatography (petroleum ether/EtOAc 90:10 to 70:30) gave (4*S*)-**26b** (183 mg, 42%) as a white solid. R_f 0.56 (petroleum ether/EtOAc 70:30). Mp 74–76 °C. $[\alpha]_D$ +28.4 (c 1.1, $CHCl_3$, 20 °C). IR (KBr) *v* 3276, 2918, 2850, 1636, 1472, 1104 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42–7.26 (m, 20H), 6.74 (d, *J* = 8.5, 1H), 4.94 (d, *J* = 9.4, 1H), 4.92 (br. s, 1H), 4.84 (br. s, 1H), 4.84 (d, *J* = 11.1, 1H), 4.79 (d, *J* = 11.9, 1H), 4.75 (d, *J* = 12.1, 1H), 4.66 (d, *J* = 11.3, 1H), 4.58 (d, *J* = 11.4, 1H), 4.62–4.44 (m, 1H), 4.49 (d, *J* = 11.9, 1H), 4.41 (d, *J* = 11.9, 1H), 4.09 (dd, *J* = 10.0, 3.7, 1H), 4.03 (dd, *J* = 12.0, 4.2, 1H), 3.99 (d, *J* = 2.3, 1H), 3.95 (t, *J* = 6.6, 1H), 3.91–3.83 (m, 2H), 3.55 (dd, *J* = 9.4, 6.1, 1H), 3.51 (dd, *J* = 9.1, 6.9, 1H), 3.62–3.44 (m, 1H), 2.02–1.96 (m, 1H), 1.92–1.88 (m, 1H), 1.68–1.55 (m, 3H), 1.49 (dt, *J* = 14.5, 7.4, 2H), 1.40–1.09 (m, 67H), 0.89 (t, *J* = 7.1, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.4, 138.4 (2C), 138.1, 137.8, 128.5–127.1 (20C), 121.8 (dd, *J* = 256, 251), 99.4, 78.9, 76.8, 74.8, 74.6, 74.1, 73.4, 72.7, 70.1, 69.4 (dd, *J* = 32, 25), 68.8, 65.2, 49.7 (dd, *J* = 33, 24), 36.0, 31.9, 29.9–29.0 (31C), 27.6 (d, *J* = 4), 25.9, 25.4, 22.6, 14.1 (2C). ^{19}F NMR (376 MHz, $CDCl_3$) δ -119.6 (dd, *J* = 253, 25, 1F), -124.0 (dd, *J* = 253, 25, 1F). MS (ESI+)

m/z 1260.9 $[M + Na]^+$. HRMS (ESI+) for $C_{78}H_{121}F_2NO_8Na^+$ $[M + Na]^+$ calcd. 1260.8952, found 1260.8923.

4.14. *1-O-(α-Galactosyl)-(2S,4R)-3,3-difluorooctadecan-1,4-diol-2-yl hexacosanamide* (4*R*)-**10a**. Galactosyl ceramide (4*R*)-***26a*** (162 mg, 0.131 mmol) was dissolved in a mixture of EtOH/$CHCl_3$ (8:2, 10 mL) and $Pd(OH)_2/C$ (110 mg, 0.157 mmol) was added to the solution. The latter was flushed with H_2 and stirred under H_2 atmosphere for 17 h. The mixture was then filtered through Celite®, and the pad was rinsed with warm EtOH and warm $CHCl_3$. Concentration of the filtrate gave a white solid which was purified by column chromatography (DCM/MeOH 90:10) to give galactosyl ceramide (4*R*)-**10a** (95 mg, 83%) as a white solid. R_f 0.22 (DCM/MeOH 90:10). Mp 155–156 °C. $[\alpha]_D$ +47.6 (c 0.46, $CHCl_3$, 20 °C). IR (KBr) *ν* 3410, 3274, 2919, 2851, 1646, 1471 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) *δ* 6.62 (br. s, 1H), 5.07 (br. s, 2H), 4.91 (br. s, 1H), 4.73 (br. s, 1H), 4.64 (br. s, 1H), 4.14 (br. s, 1H), 4.05 (br. s, 1H), 3.91 (br. s, 1H), 3.62–3.88 (m, 7H), 2.07–2.32 (m, 2H), 1.59 (br. s, 4H), 1.17–1.40 (m, 68H), 0.89 (t, *J* = 7.0, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 174.8, 122.2 (dd, *J* = 253, 246), 99.2, 76.9, 70.5, 70.3, 70.2, 68.6, 65.3, 62.1, 50.4, 36.6, 32.0, 31.9, 30.0–29.3 (32C), 25.8, 22.7, 14.1 (2C). ^{19}F NMR (376 MHz, $CDCl_3$) *δ* -118.9 (d, *J* = 258, 1F), -119.8 (d, *J* = 258, 1F). MS (CI+) m/z 879.1 $[M + H]^+$. MS (CI-) m/z 877.1 $[M – H]^-$. HRMS (ESI+) for $C_{50}H_{98}F_2NO_8$ $[M + H]^+$ calcd. 878.7255, found 878.7247. Elemental Analysis Calcd. C, 65.36; H, 11.13; N, 1.52 (**10a**+2.25H_2O); Found C, 64.95; H, 10.80; N, 1.37.

4.15. *1-O-(α-Galactosyl)-(2S,4S)-3,3-difluorooctadecan-1,4-diol-2-yl hexacosanamide* (4*S*)-**10b**. Galactosyl ceramide (*4S*)-**26b** (49 mg, 0.040 mmol) was dissolved in a mixture of EtOH/$CHCl_3$ (8:2, 3 mL) and $Pd(OH)_2/C$ (33 mg, 0.048 mmol) was added to the solution. The latter was flushed with H_2 and stirred under H_2 atmosphere for 18 h. The mixture was then filtered through Celite, and the pad was rinsed with warm EtOH and warm $CHCl_3$. Concentration of the filtrate gave a white solid which was purified by column chromatography ($CHCl_3$/MeOH 95:5 then 90:10) to give galactosyl ceramide (4*S*)-**10b** (27 mg, 77%) as a white solid. R_f 0.28 ($CHCl_3$/MeOH 90:10). Mp 175–179 °C. $[\alpha]_D$ +32.3 (c 0.4, THF, 20 °C). IR (KBr) *ν* 3417, 2915, 2850, 1653, 1468, 1076 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$/CD_3OD 2:1) *δ* 4.70 (d, *J* = 3.6, 1H), 4.38 (m, 1H), 3.81 (m, 1H), 3.74 (d, *J* = 3.0, 1H), 3.66–3.45 (m, 6H), 3.36 (dd, *J* = 23.2, 8.8, 1H), 2.09 (t, *J* = 7.5, 2H), 1.52–1.23 (m, 4H), 1.17–1.00 (m, 68H), 0.67 (t, *J* = 6.4, 6H). ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD 2:1) *δ* 176.6,

99.6, 76.9, 70.5, 69.8, 69.3, 68.6, 64.1, 61.3, 35.5, 31.5, 29.6–28.4 (31C), 27.2, 25.4, 25.3, 22.2, 13.5 (2C) [loss of 2C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, $CDCl_3/CD_3OD$ 2:1) δ -122.0 (dd, J = 254, 24, 1F), -125.7 (dd, J = 254, 23, 1F). MS (CI+) m/z 879.2 $[M + H]^+$. MS (CI-) m/z 877.0 $[M – H]^-$. HRMS (ESI+) for $C_{50}H_{98}F_2NO_8$ $[M + H]^+$ calcd. 878.7255, found 878.7242. Elemental Analysis calcd. C, 66.67; H, 11.13; N, 1.55 (**10b**+1.25H_2O); Found C, 66.78; H, 11.19; N, 1.40.

4.16. *(3R)-4-Benzyloxy-1-bromo-3-tert-butyldimethylsilyloxy-1,1,2,2-tetrafluorobutane* **29**. To alcohol **28**[152] (8.35 g, 25.2 mmol) dissolved in DMF (120 mL) were added TBDMSCl (4.56 g, 30.2 mmol), imidazole (5.15 g, 75.6 mmol) and DMAP (309 mg, 2.52 mmol). The mixture was heated at 50 °C for 4 days and then quenched with brine (250 mL). The aqueous layer was extracted with Et_2O (3 × 250 mL) and combined organic layers were dried ($MgSO_4$), filtered and concentrated to give an orange oil. Column chromatography (petroleum ether/Et_2O 99:1 to 70:30) gave compound **29** (6.71 g, 60%) as a colourless oil and starting alcohol **28** (2.78 g, 33%) as a yellow oil. R_f 0.74 (petroleum ether/Et_2O 60:40). $[\alpha]_D$ +4.9 (c 1.0, $CHCl_3$, 20 °C). IR (neat) ν 2931, 2860, 1254, 1160, 838 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7. 29 (m, 5H), 4.57 (d, J = 11.8, 1H), 4.52 (d, J = 11.8, 1H), 4.41 (dddd, J = 12.1, 9.6, 7.0, 2.4, 1H), 3.79 (dd, J = 10.1, 2.6, 1H), 3.57 (dd, J = 9.5, 7.6, 1H), 0.89 (s, 9H), 0.11 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.5, 128.4 (2C), 127.8, 127.7 (2C), 119.2–110.1 (m, 2C), 73.6, 71.8 (t, J = 26), 70.4, 25.6 (3C), 18.1, -4.5, -5.3. ^{19}F NMR (376 MHz, $CDCl_3$) δ -61.2 (dd, J = 178, 7, 1F), -62.0 (dd, J = 178, 5, 1F), -113.4 (ddd, J = 272, 9, 5, 1F), -118.6 (ddd, J = 272, 13, 8, 1F). MS (CI+) m/z 464.0 $[M(^{81}Br) + NH_4]^+$. HRMS (ESI+) for $C_{17}H_{25}O_2BrF_4SiNa^+$ $[M + Na]^+$ calcd. 467.0636, found 467.0630.

4.17. *(2R)-1-Benzyloxy-2-tert-butyldimethylsilyloxy-3,3,4,4-tetrafluorooctadecan-5-ol* **30**. Bromide **29** (2.40 g, 5.39 mmol) and tetradecanal (2.82 g, 13.3 mmol) were independently dissolved in DCM and filtered under nitrogen through Na_2SO_4. The two filtrates were dried under high vacuum for several hours. The resultant dried tetradecanal was dissolved in THF (9 mL) and added to the dried bromide **29**. In a separate flask, to THF (2 mL) at -74 °C was added MeLi solution (1.35 M in Et_2O, 9.59 mL, 12.9 mmol) and then dropwise the THF mixture of bromide **29** and aldehyde. The reaction was then stirred at -74 to -69 °C for 45 min and to -55 to -50 °C for another 1.5 h. The mixture was quenched with aq. NH_4Cl (sat., 21 mL) then allowed to warm at r.t. over 20 min. H_2O (42 mL) was added and the aqueous layer

was extracted with EtOAc (3 × 100 mL). The combined organic phases were dried ($MgSO_4$), filtered and concentrated to give a yellow oil. Column chromatography (petroleum ether/Et_2O 100:0 to 95:5) gave alcohols **30** (2.54 g, 81%) as a colourless oil and alkene **31** (186 mg, 10%).

Alcohols **30a/30b** were run as a mixture in the next step, but for analytical characterization, the two diastereoismomers were separated by flash chromatography (petroleum ether/Et_2O 90:10).

4.17.1. *Data for diasteroisomer **30a***: R_f 0.09 (petroleum ether/Et_2O 95:5). $[\alpha]_D$ +10.1 (c 1.0, $CHCl_3$, 20 °C). IR (KBr) *v* 3439, 2927, 2855, 1497, 1257, 1107 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.28 (m, 5H), 4.56 (d, *J* = 11.8, 1H), 4.52 (d, *J* = 11.7, 1H), 4.35 (m, 1H), 3.98 (m, 1H), 3.81 (d, *J* = 10.0, 1H), 3.62 (br. dd, *J* = 9.7, 8.2, 1H), 2.78 (d, *J* = 8.2, 1H), 1.74 (m, 1H), 1.66–1.52 (m, 2H), 1.30 (s, 21H), 0. 97–0.88 (m, 12H), 0.17 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.5, 128.4 (2C), 127.8 (2C), 119.9–112.8 (m, 2C), 73.5, 73.2 (dd, *J* = 29, 23), 71.0 (t, *J* = 26), 70.3 (t, *J* = 6), 31.9, 29.9–29.1 (9C), 25.9 (3C), 25.7, 25.4, 22.7, 18.2, 14.1 (3C), –4.8, –4.9. ^{19}F NMR (376 MHz, $CDCl_3$) δ -116.6 (dd, *J* = 277, 11, 1F), -117.7 (d, *J* = 278, 1F), -119.4 (dt, *J* = 278, 9, 1F), -126.5 (dddd, *J* = 277, 12, 8, 4, 1F). MS (CI+) m/z 596.4 $[M + NH_4]^+$. HRMS (ESI+) for $C_{31}H_{54}O_3F_4SiNa^+$ $[M + Na]^+$ calcd. 601.3671, found 601.3662.

4.17.2. *Data for diastereoisomer **30b***: R_f 0.19 (petroleum ether/Et_2O 95:5). $[\alpha]_D$ –3.8 (c 1.0, $CHCl_3$, 20 °C). IR (KBr) *v* 3430, 2932, 2860, 1258, 1109 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.28 (m, 5H, H_{Ar}), 4.56 (d, *J* = 11.7, 1H), 4.49 (d, *J* = 11.7, 1H), 4. 36–4. 24 (m, 2H), 3. 99–3. 86 (m, 1H), 3.79 (app. d, *J* = 10.4, 1H), 3.69 (br. dd, *J* = 10.4, 8.3, 1H), 1.70 (m, 1H), 1.65–1.55 (m, 2H), 1.50–1.14 (m, 21H), 0.99–0.81 (m, 12H), 0.18 (s, 3H), 0.16 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.5, 128.4 (2C), 127.7 (2C), 120.1-113.0 (m, 2C), 74.1 (dd, *J* = 33, 23), 73.6, 70.0 (t, *J* = 7), 68.0 (dd, *J* = 28, 22), 31.9, 29.8–29.3 (9C), 27.8 (3C), 25.5, 25.2, 22.7, 18.1, 14.1, -4.7, -5.3. ^{19}F NMR (376 MHz, $CDCl_3$) δ -117.2 (dq, *J* = 279, 11, 1F), -121.6 (dq, *J* = 279, 10, 1F), -122.1 (br. dt, *J* = 273, 11, 1F), -130.6 (ddt, *J* = 273, 22, 11, 1F). MS (CI) m/z 596.4 $[M + NH_4]^+$. HRMS (ESI+) for $C_{31}H_{55}O_3F_4Si^+$ $[M + H]^+$ calcd. 579.3851, found 579.3842.

4.17.3. *Data for (3R)-4-Benzyloxy-3-tert-butyldimethylsilyloxy-1,1,2-trifluorobutene* **31***:* ^1H NMR (300 MHz, $CDCl_3$) δ 7. 51–7. 35 (m, 5H), 4.75-4.56 (m, 3H), 3.80-3.61 (m, 2H), 0.90 (s, 9H), 0.09 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.0, 128.6 (2C), 127.9, 127.7 (2C), 73.6, 70.2, 65.9 (dt, *J* = 2, 21), 25.7 (3C), 18.2, -5.0, -4.9 [loss of 2C related to fluorine due to

bad relaxation]. ^{19}F NMR (282 MHz, $CDCl_3$) δ -102.3 (dd, J = 79, 32, 1F), -120.2 (dd, J = 115, 79, 1F), -189.3 (dd, J = 32, 115, 1F). MS (ESI+) m/z 369.1 $[M + Na]^+$. HRMS (ESI+) for $C_{17}H_{25}F_3O_2SiNa^+$ $[M + Na]^+$ calcd. 369.1474, found 369.1472.

4.18. *(2R)-1-Benzyloxy-2-tert-butyldimethylsilyloxy-3,3,4,4-tetrafluorooctadecane* **32**. Alcohols **30a** and **30b** (1.68 g, 2.91 mmol) were dissolved in dichloroethane (DCE) (12 mL) and treated with thiocarbonyldiimidazole (TCDI) (1.56 g, 8.73 mmol). The mixture was stirred at r.t. for 18 h then concentrated under reduced pressure to give an orange residue. Flash chromatography (petroleum ether/Et_2O 90:10) gave thiocarbamate intermediates, *O-((2R)-1-Benzyloxy-2-tert-butyldimethylsilyloxy-3,3,4,4-tetrafluorooctadecan-5-yl)-1H-imidazole-1-carbothioate*, (1.89 g, 95%) as a colourless oil.

For analytical characterization, the two diastereoismomers of thiocarbamate intermediates were separated by flash chromatography (petroleum ether/Et_2O 90:10).

4.18.1. *Data for diastereoisomer* ***a***: $[\alpha]_D$ +12.2 (c 1.0, $CHCl_3$, 20 °C). IR (KBr) ν 2927, 2855, 1464, 1395, 1286, 1116 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (br. t, J = 0.9, 1H), 7.64 (t, J = 1.5, 1H), 7.40–7.28 (m, 5H), 7.06 (dd, J = 1.7, 0.8, 1H), 6.20 (dtd, J = 13.5, 8.3, 4.7, 1H), 4.55 (d, J = 12.0, 1H), 4.50 (d, J = 11.9, 1H), 4.34 (dddd, J = 12.4, 9.7, 6.9, 3.1, 1H), 3.77 (dd, J = 10.1, 1.9, 1H), 3.56 (dd, J = 9.7, 7.4, 1H), 2.11–1.91 (m, 2H), 1.56–1.18 (m, 22H), 0.98–0.82 (m, 12H), 0.19–0.04 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.0, 137.5, 137.0, 130.9, 128.3 (2C), 127.7 (3C), 118.1, 78.2 (dd, J = 30, 24), 73.5, 71.9 (t, J = 26), 70.2, 31.9, 29.8–29.0 (8C), 27.8, 25.6, 24.6, 22.7, 18.1, 14.1 (3C), –4.5, –5.3 [loss of 2C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, $CDCl_3$) δ -117.7 (dt, J = 280, 13, 1F), -118.1 (ddd, J = 282, 15, 9, 1F), -119.8 (dtd, J = 282, 14, 6, 1F), -121.1 (dtd, J = 280, 15, 15, 6, 1F). MS (CI+) m/z 689.0 $[M + H]^+$. HRMS (ESI+) for $C_{35}H_{56}F_4N_2O_3SSiNa^+$ $[M + Na]^+$ calcd. 711.3609, found 711.3604.

4.18.2. *Data for diasteroisomer* ***b***: $[\alpha]_D$ –6.8 (c 0.9, $CHCl_3$, 20 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (t, J = 0.9, 1H), 7.65 (dd, J = 1.7, 1.3, 1H), 7.39–7. 28 (m, 5H), 7.07 (dd, J = 1.7, 0.8, 1H), 6.34 (tt, J = 11.2, 5.8, 1H), 4.56 (d, J = 11.9, 1H), 4.50 (d, J = 11.8, 1H), 4.33 (m, 1H), 3.76 (br. d, J = 9.5, 1H), 3.59 (br. dd, J = 9.3, 7.9, 1H), 2.13–1.88 (m, 2H), 1.50–1.15 (m, 22H), 0.98–0.83 (m, 12H), 0.13 (s, 3H), 0.10 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.4, 137.5, 137.1, 131.0, 128.3 (2C), 127.7 (3C), 120.0–111.2 (m, 2C), 118.1, 78.5 (dd, J = 27, 21), 73.5, 72.5 (t, J = 25), 70.3, 31.9, 29.8–29.0 (8C), 27.9, 25.6, 24.6, 22.6, 18.1, 14.1 (3C), –4.8, -5.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -118.2 (ddd, J = 280, 11, 7, 1F), -118.8 (ddd,

J = 271, 10, 7, 1F), -119.6 (m, 1F), -120.3 (m, 1F). MS (CI) m/z 689.2 $[M + H]^+$. HRMS (ESI+) for $C_{35}H_{56}F_4N_2O_3SSiNa^+$ $[M+Na]^+$ calcd. 711.3609, found 711.3605.

To a mixture of thiocarbamate intermediates (3.82 g, 5.55 mmol) dissolved in toluene (60 mL, degassed by bubbling of argon) was added AIBN (911 mg, 5.55 mmol). The reaction was stirred at 110 °C for 5 min then cooled at r.t. prior adding a solution of Bu_3SnH (5.97 mL, 22.2 mmol) in toluene (52 mL, degassed). The resultant mixture was stirred at 110 °C for 40 min then concentrated to give a yellow oil. Column chromatography (pentane) gave alkane **32** (2.92 g, 94%) as a colourless oil.

4.18.3 Data for **32**: R_f 0.74 (petroleum ether/Et_2O 95:5). $[\alpha]_D$ +4.5 (c 1.0, $CHCl_3$, 20.0 °C). IR (neat) ν 2297, 2856, 1465, 1128 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.28 (m, 5H), 4.55 (d, J = 11.8, 1H), 4.52 (d, J = 11.8, 1H), 4.28 (m, 1H), 3.78 (d, J = 10.3, 1H), 3.54 (dd, J = 9.6, 8.6, 1H), 2.12–1.91 (m, 2H), 1.56–1.48 (m, 2H), 1.40–1.20 (m, 22H), 0.95–0.86 (m, 12H), 0.14–0.07 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.9, 128.3 (2C), 127.7 (2C), 127.6, 123.5–112.5 (m, 2C), 73.5, 72.4 (t, J = 26 Hz), 70.8, 32.0, 31.5 (t, J = 23), 29.1–29.9 (9C), 25.7, 22.7, 20.3, 18.2, 14.1 (3C), –4.6, –5.2. ^{19}F NMR (376 MHz, $CDCl_3$) δ -113.5 (dddd, J = 264, 26, 12, 3, 1F), -114.5 (ddd, J = 264, 26, 13, 1F), -119.9 (ddd, J = 276, 12, 5 , 1F), -121.5 (ddt, J = 276, 10, 5, 1F). MS (CI+) m/z 580.5 $[M + NH_4]^+$. HRMS (ESI+) for $C_{31}H_{54}O_2F_4SiNa^+$ $[M + Na]^+$ calcd. 585.3721, found 585.3714.

4.19. *(2R)-1-Benzyloxy-2-trifluoromethanesulfonyloxy-3,3,4,4-tetrafluorooctadecane* **33**. Alkane **32** (2.85 g, 5.05 mmol) was dissolved in THF (56 mL) and treated with TBAF.$3H_2O$ (3.98 g, 12.6 mmol). The mixture was stirred at r.t. for 40 min then concentrated to give a green oil. Column chromatography (petroleum ether/Et_2O 95:5 to 80:20) gave the alcohol intermediate (2.23 g, 98%),

4.19.1. Data for intermediate alcohol *(2R)-1-Benzyloxy-3,3,4,4-tetrafluorooctadecan-2-ol*, as a colourless oil. R_f 0.29 (petroleum ether/Et_2O 95:5). Mp 40–43 °C. $[\alpha]_D$ +2.0 (c 0.9, $CHCl_3$, 20 °C). IR (KBr) ν 3432, 2917, 2850, 1100 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.42–7.30 (m, 5H), 4.63 (d, J = 12.3, 1H), 4.60 (d, J = 12.3, 1H), 4.28 (m, 1H), 3.80 (dt, J = 10.1, 2.5, 1H), 3.73 (dd, J = 9.8, 6.8, 1H), 2.81 (d, J = 5.5, 1H), 2.17–1.92 (m, 2H), 1.58–1.50 (m, 2H), 1.38–1.23 (m, 22 H), 0.89 (t, J = 6.5, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.3, 128.5 (2C), 128.0, 127.8 (2C), 122.2–113.5 (m, 2C), 73.6, 68.7 (dd, J = 27, 23), 68.0, 31.9, 31.1 (t, J = 23), 29.0–29.9 (9C), 22.7, 20.4, 14.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -114.3 (dddd, J = 264,

23, 16, 4, 1F), -115.1 (dddd, J = 264, 22, 15, 5, 1F), -122.9 (d, J = 274, 1F), -126.1 (ddd, J = 275, 17, 5, 1F). MS (CI+) m/z 466.3 $[M + NH_4]^+$. HRMS (ESI+) for $C_{25}H_{40}F_4O_2Na^+$ $[M + Na]^+$ calcd. 471.2857, found 471.2857.

Intermediate alcohol (2.11 g, 4.70 mmol) was dissolved in DCM (16 mL) and pyridine (761 µL, 9.41 mmol) was added prior cooling the solution at -40 °C. Tf_2O (1M in DCM, 5.65 mL, 5.65 mmol) was then added. The mixture was stirred at -40 °C for 1 h, then warmed to -10 °C and stirred for another 1.5 h before being quenched with aq. NH_4Cl (sat., 60 mL). The aqueous layer was extracted with Et_2O (3 × 120 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated to give a yellow oil. Column chromatography (petroleum ether /Et_2O 100:0 to 97:3) gave triflate **33** (2.47 g, 91%) as a colourless oil.

4.19.2. Data for **33**: $[\alpha]_D$ +5.4 (c 0.9, $CHCl_3$, 20.0 °C). IR (KBr) ν 2926, 2856, 1424, 1213, 1142, 937 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.45–7.29 (m, 5H), 5.39 (dddd, J = 15.1, 8.7, 6.6, 2.2, 1H), 4.64 (s, 2H), 3.96 (dd, J = 11.8, 1.3 Hz, 1H), 3.84 (dd, J = 11.5, 8.8, 1H), 2.04 (br. ddd, J = 27.0, 17.9, 7.7, 2H), 1.60-1.56 (m, 2H), 1.43–1.21 (m, 22H), 0.92 (t, J = 6.9, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 136.6, 128.5 (2C), 128.1, 127.8 (2C), 124.8–113.0 (m, 2C), 118.4 (q, J = 315), 81.2, 73.7, 66.0-65.8 (m), 31.9, 30.2 (t, J = 23), 29.0–29.7 (9C), 22.7, 20.1 (t, J = 3), 14.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -74.0 (d, J = 5, 3F), -113.2 (qd, J = 18, 10, 2F), -116.3 (d, J = 282, 1F), -121.9 (ddd, J = 282, 13, 10, 1F). MS (CI+) m/z 598.4 $[M + NH_4]^+$. HRMS (ESI+) for $C_{26}H_{39}F_7O_4SNa^+$ $[M + Na]^+$ calcd. 603.2355, found 603.2355.

4.20. *(2S)-2-Azido-1-benzyloxy-3,3,4,4-tetrafluorooctadecane* **34**. Triflate **33** (2.47 g, 4.25 mmol) was dissolved in DMF (30 mL) and the solution was cooled to 0 °C. NaN_3 (1.38 g, 21.3 mmol) was then added and the mixture was stirred at 0 °C for 6 h, then heated to 50 °C and stirred for another 14 h before being quenched with brine (35 mL). The aqueous layer was extracted with Et_2O (3 × 55 mL) and the combined organic layers dried ($MgSO_4$), filtered and concentrated to give an oil. Flash chromatography (pentane/Et_2O 100:0 to 80:20) afforded azide **34** (1.54 g, 77%) as a yellow oil, alkene (*Z*)-**35** (148 mg, 8%) as a white solid and alkene (*E*)-**35** (195 mg, 10%) as a white solid.

4.20.1. *Data for **34***: R_f 0.28 (petroleum ether/Et_2O 99:1). $[\alpha]_D$ +3.1 (c 0.96, $CHCl_3$, 20 °C). IR (KBr) ν 2925, 2854, 2111, 1455, 1114 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.47–7.30 (m, 5H), 4.67 (d, J = 11.8, 1H), 4.62 (d, J = 11.8, 1H), 4.18–4.01 (m, 1H), 3.96 (dd, J = 10.3, 2.7, 1H), 3.74 (dd, J = 11.0, 8.1, 1H), 2.16–1.90 (m, 2H), 1.59 (app. dq, J = 7.6, 7.0, 2H), 1.45–1.20 (m, 22H), 0.93 (t, J = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.2, 128.5 (2C),

127.9, 127.6 (2C), 124.2–112.2 (m, 2C), 73.6, 67.2, 60.7 (dd, J = 25, 23), 31.9, 30.7 (t, J = 23), 29.7–28.8 (9C), 22.7, 20.3 (t, J = 4), 14.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -113.4 (dtd, J = 266, 19, 10, 1F), -114.2 (dtd, J = 266, 19, 19, 8, 1F), -118.2 (dt, J = 275, 10, 1F), -119.2 (ddd, J = 275, 14, 10, 1F). MS (CI+) m/z 491.3 $[M + NH_4]^+$. HRMS (ESI+) for $C_{25}H_{39}F_4N_3ONa^+$ $[M + Na]^+$ calcd. 496.2922, found 496.2928.

4.20.2. *Data for (Z)-1-Benzyloxy-3,3,4,4-tetrafluorooctadec-1-ene (Z)-**35***: R_f 0.28 (petroleum ether/Et_2O 99:1). Mp 35–37 °C. IR (KBr) ν 2920, 2850, 1675, 1457, 1378 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.32 (m, 5H), 6.44 (dt, J = 7.2, 1.8, 1H), 4.98 (s, 2H), 4.60 (td, J = 14.9, 7.2, 1H), 2.03 (ddd, J = 26.5, 18.1, 7.8, 2H), 1.61 (dq, J = 8.0, 7.2, 2H), 1.44–1.26 (m, 22H), 0.94 (t, J = 6.3, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.7 (t, J = 5), 136.3, 128.6 (2C), 128.3, 127.2 (2C), 124.6–112.2 (m, 2C), 95.0 (t, J = 25), 75.4, 31.9, 30.2 (t, J = 23), 29.8–29.0 (9C), 22.7, 20.6, 14.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -107.8 (d, J = 15, 2F), -116.3 (t, J = 18, 2F). MS (CI) m/z 448.3 $[M + NH_4]^+$. HRMS (ESI+) for $C_{25}H_{38}F_4ONa^+$ $[M + Na]^+$ calcd. 453.2751, found 453.2762.

4.20.3. *Data for (E)-1- Benzyloxy-3,3,4,4-tetrafluorooctadec-1-ene (E)-**35***: R_f 0.50 (petroleum ether/Et_2O 99:1). Mp 47–48 °C. IR (KBr) ν 2919, 2851, 1659, 1472, 1189 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46–7.32 (m, 5H), 7.05 (dt, J = 12.8, 2.0, 1H), 5.06 (q, J = 12.4, 1H), 4.86 (s, 2H), 2.00 (ddd, J = 26.1, 18.8, 7.8, 2H), 1.62-1.58 (m, 2H), 1.44–1.23 (m, 22H), 0.93 (t, J = 7.0, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.8 (t, J = 11), 135.5, 128.7 (2C), 128.4, 127.6 (2C), 124.6–112.8 (m, 2C), 94.3 (t, J = 24), 71.9, 31.9, 30.3 (t, J = 24), 29.8–29.1 (9C), 22.7, 20.6, 14.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -109.3 (d, J = 12, 2F), -115.7 (m, J = 18, 2F). MS (CI+) m/z 448.3 $[M + NH_4]^+$. HRMS (ESI+) for $C_{25}H_{38}F_4ONa^+$ $[M + Na]^+$ calcd. 453.2751, found 453.2762.

4.21. *(2S)-1-Benzyloxy-3,3,4,4-tetrafluorooctadecan-2-amine* **37**. Azide **34** (1.396 g, 2.95 mmol) was dissolved in mixture of THF/H_2O (5:1, 54 mL) and PPh_3 (1.161 g, 4.43 mmol) was added. The mixture was stirred at 60 °C for 13 h and concentrated to give a white solid. Flash chromatography (petroleum ether/EtOAc 100:0 to 94:6) gave amine **37** (1.23 g, 93%) as a white solid. R_f 0.38 (petroleum ether/EtOAc 80:20). Mp 34–37 °C. $[\alpha]_D$ –9.3 (c 0.98, $CHCl_3$, 20 °C). IR (KBr) ν 3432, 2918, 2851 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42–7.28 (m, 5H), 4.58 (s, 2H), 3.79 (app. td, J = 6.9, 6.2, 1H), 3.68–3.52 (m, 2H), 2.03 (app. dddd, J = 26.7, 17.6, 7.6, 1.3, 2H), 1.56 (dt, J = 14.9, 7.4, 2H), 1.40–1.20 (m, 22H), 0.89 (t, J = 6.4, 3H) NH_2 not observed. ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.8, 128.4 (2C), 127.8, 127.7 (2C),

125.4–112.0 (m, 2C), 73.5, 69.0, 52.3 (t, J = 23), 31.9, 30.7 (t, J = 23), 29.8–29.0 (9C), 22.7, 20.3, 14.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -113.4 (t, J = 16, 2F), -120.8 (d, J = 271, 1F), -121.9 (dd, J = 271, 13, 1F). MS (CI+) m/z 448.3 $[M + H]^+$.HRMS (ESI+) for $C_{25}H_{42}F_4NO^+$ $[M + H]^+$ calcd. 448.3197, found 448.3205.

4.22. *(2S)-2-(N-(Triphenylphosphoranylidene))amino-1-benzyloxy-3,3,4,4-tetrafluoro-octadecane* **36**. Azide **34** (97 mg, 0.21 mmol) was dissolved in THF (3 mL) and PPh_3 (81 mg, 0.31 mmol) was added. The mixture was stirred at r.t. for 2 h and concentrated to give a white solid. Flash chromatography (EtOAc) gave phosphoranyl **36** (130 mg, 90%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75-7.61 (m, 6H), 7.54-7.32 (m, 10H), 7.26-7.18 (m, 2H), 7.05-6.92 (m, 2H), 4.25 (d, J = 11.3, 1H), 4.17 (d, J = 11.2, 1H), 3.93 (d, J = 8.6, 1H), 3.71 (t, J = 8.6, 1H), 3.63 (m, 1H), 2.13-1.88 (m, 4H), 1.61-1.39 (m, 2H), 1.38-1.08 (m, 20H), 0.91 (t, J = 6.9, 3H). ^{31}P NMR (121 MHz, $CDCl_3$) δ 14.3. MS (CI+) m/z 708.3 $[M + H]^+$. HRMS (ESI+) for $C_{43}H_{55}F_4NOP^+$ $[M + H]^+$ calcd. 708.3949, found 708.3948.

4.23. *(2S)-1-Benzyloxy-3,3,4,4-tetrafluorooctadecan-2-yl hexacosanamide* **38**. The amine **37** (943 mg, 2.11 mmol) in DCM (100 mL) was treated with cerotic acid (936 mg, 2.53 mmol), PyBOP (2.42 g, 4.64 mmol) and Et_3N (0.59 mL, 4.22 mmol). The mixture was stirred at reflux for 21 h and then diluted with DCM (100 mL). The organic layer was washed with H_2O (100 mL) and brine (100 mL), dried ($MgSO_4$), filtered and concentrated to give a white solid. Flash chromatography (petroleum ether/Et_2O 98:2 to 0:100) gave amide **38** (1.06 g, 61%) as a white solid. R_f 0.53 (petroleum ether/EtOAc 80:20). Mp 78-79 °C. $[\alpha]_D$ +11.6 (c 1.0, $CHCl_3$, 20 °C). IR (KBr) ν 3330, 2917, 2849, 1659, 1540, 1499 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42–7.27 (m, 5H), 5.94 (d, J = 9.9, 1H), 4.94 (dddd, J = 18.2, 14.2, 9.9, 4.1, 1H), 4.54 (s, 2H), 3.82 (dd, J = 10.4, 4.0, 1H), 3.66 (br. d, J = 10.1, 1H), 2.21 (t, J = 7.6, 2H), 2.11–1.87 (m, 2H), 1.65–1.61 (m, 2H), 1.56–1.52 (m, 2H), 1.42–1.15 (m, 66H), 0.89 (t, J = 6.4, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 137.5, 128.4 (2C), 127.8, 127.7 (2C), 73.3, 67.1, 47.9 (dd, J = 26, 22), 36.6, 31.9, 30.5 (t, J = 23), 29.9-28.9 (31C), 25.5, 22.7, 20.4, 14.1 (2C) [loss of 2C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, $CDCl_3$) δ -113.6 (ddt, J = 265, 27, 10, 1F), -115.3 (ddtd, J = 265, 27, 11, 4, 1F), -117.9 (dt, J = 274, 9, 1F), -119.7 (m, 1F). MS (CI+) m/z 827.0 $[M + H]^+$. HRMS (ESI+) for $C_{51}H_{91}F_4NO_2Na^+$ $[M + Na]^+$ calcd. 848.6884, found 848.6879.

4.24. *(2S)-3,3,4,4-Tetrafluorooctadecan-1-ol-2-yl hexacosanamide* **39**. Amide **38** (633 mg, 0.77 mmol) was dissolved in THF (11 mL) and $Pd(OH)_2/C$ (20% wt, 161 mg, 0.23 mmol) was added to the solution. The reaction mixture was flushed with H_2 then stirred under H_2 atmosphere for 3 h before being filtered through Celite®. The pad was rinsed with warm THF and the filtrate was concentrated to give a white solid. Flash chromatography (DCM/MeOH 99:1 to 80:20) gave ceramide **39** (528 mg, 94%) as a white solid. R_f 0.27 (petroleum ether/EtOAc 70:30). Mp 89–90 °C. $[\alpha]_D$ +3.8 (c 0.5, THF, 20 °C). IR (neat) *ν* 3427, 2921, 2850, 1661 cm^{-1}. ^1H NMR (400 MHz, THF-d_8) δ 7.25 (d, J = 9.8, 1H), 4.70 (m, 1H), 3.91 (t, J = 6.3, 1H), 3.76 (m, 1H), 3.64 (m, 1H), 2.17 (t, J = 7.4, 2H), 2.13–1.86 (m, 2H), 1.65–1.56 (m, 2H), 1.56-1.51 (m, 2H), 1.39–1.24 (m, 66H), 0.89 (t, J = 7.0, 6H). ^{13}C NMR (100 MHz, THF-d_8) δ 173.0, 60.2, 51.4 (dd, J = 26, 21), 36.5, 32.9, 31.7 (t, J = 23), 30.9–30.1 (31C), 26.5, 23.6, 21.5, 14.5 (2C) [loss of 2C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, THF-d_8) δ -114.6 (ddd, J = 262, 29, 10, 1F), -116.7 (dddd, J = 262, 29, 10, 4, 1F), -119.1 (dd, J = 271, 8, 1F), -123.9 (dd, J = 271, 20, 1F). MS (CI+) m/z 736.8 $[M + H]^+$. HRMS (ESI+) for $C_{44}H_{86}F_4NO_2^+$ $[M + H]^+$ calcd. 736.6589, found 736.6601.

4.25. *1-O-(2,3,4,6-Tetra-O-benzyl-α-galactosyl)-(2S)-3,3,4,4-tetrafluorooctadecan-1-ol-2-yl hexacosanamide* **40α**. In the Dark, $SnCl_2$ (89 mg, 0.47 mmol), $AgClO_4$ (98 mg, 0.47 mmol) and ground 4Å molecular sieves (685 mg) were combined in THF (1.1 mL) and stirred at r.t. for 90 min. In parallel, ceramide **39** (116 mg, 0.16 mmol) was dissolved in THF (2.7 mL) and added to a solution of fluoro-galactosyl donor **25**[162] (128 mg, 0.24 mmol) dissolved in THF (3 mL). Then, the solution containing **25** and **39** was added, *via* cannula, to the mixture of Lewis acids beforehand cooled to 0°C and stirring was maintained, in the dark, for 20 min. The mixture was warmed to r.t., stirred for 2 h and then filtered through Celite®, which was rinsed with EtOAc (~40 mL). The filtrate was washed with aq. $NaHCO_3$ (sat., 5 × 5 mL), dried ($MgSO_4$), filtered and concentrated to give a white solid. Flash chromatography (petroleum ether/EtOAc 97:3 to 70:30) gave **40α** (112 mg, 57%) as a white solid. R_f 0.53 (petroleum ether/EtOAc 70:30). *Data for* **40α***:* Mp 89–90 °C. $[\alpha]_D$ +35.6 (c 1.1, $CHCl_3$, 20 °C). IR (KBr) *ν* 3427, 2921, 2850, 1661 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.48–7.16 (m, 20H), 6.44 (d, J = 9.7, 1H), 4.93 (d, J = 11.5, 1H), 4.81 (d, J = 11.9, 1H), 4.90-4.80 (m, 2H), 4.79 (d, J = 12.1, 1H), 4.73 (d, J = 12.1, 1H), 4.64 (d, J = 11.9, 1H), 4.56 (d, J = 11.5, 1H), 4.49 and 4.40 (AB syst. d, J = 12.0, 2H), 4.10 (dd, J = 10.9, 1.7, 1H), 4.05 (dd, J = 8.0, 1.9, 1H), 4.00 (t, J = 6.3, 1H), 3.92-3.86 (m, 2H), 3.71 (d, J = 11.8, 1H), 3.56 (dd, J = 9.5,

6.8, 1H), 3.40 (dd, *J* = 9.5, 5.8, 1H), 2.19–1.80 (m, 4H), 1.64–1.44 (m, 4H), 1.41–1.06 (m, 66H), 0.88 (t, *J* = 6.2, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 138.6, 138.4 (2C), 137.7, 128.6–127.2 (20C), 100.0, 78.7, 76.7, 74.8, 74.6, 73.5, 73.3, 73.1, 70.1, 69.3, 67.9, 48.1 (dd, *J* = 27, 21), 36.2, 31.9, 30.5 (t, *J* = 23), 29.9–28.9 (31C), 25.3, 22.7, 20.4, 14.1 (2C) [loss of 2C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, $CDCl_3$) δ -113.5 (ddt, *J* = 263, 27, 9, 1F), -115.3 (m, 1F), -117.1 (br. d, *J* = 274, 1F), -119.7 (dd, *J* = 274, 17, 1F). HRMS (MALDI+) for $C_{78}H_{119}F_4NO_7Na^+$ $[M + Na]^+$ calcd 1280.8815, found 1280.8786.

4.26. *1-O-α-Galactosyl-(2S)-3,3,4,4-tetrafluorooctadecan-1-ol-2-yl hexacosanamide* **11**. Protected Galactosyl ceramide **40α** (100 mg, 79 µmol) was dissolved in EtOH (4.6 mL) and $CHCl_3$ (1.2 mL) prior adding $Pd(OH)_2$/C (20%, 67 mg, 95 µmol) to the solution. The latter was flushed with H_2 then stirred under H_2 atmosphere for 15 h before being filtered through a pad of Celite®. The pad was rinsed with warm EtOH and warm $CHCl_3$, and the solution was concentrated to give a white solid. Flash chromatography (DCM/MeOH 90:10) gave tetrafluorinated galactosyl ceramide **11** (60 mg, 85%) as a white solid. R_f 0.16 (DCM/MeOH 90:10). Mp 146–148 °C. $[\alpha]_D$ +47.1 (c 0.6, $CHCl_3$, 20 °C). IR (KBr) *v* 3433, 2920, 2851, 1651, 1469 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.20 (d, *J* = 9.9, 1H), 4.98 (m, 1H), 4.95 (d, *J* = 3.5, 1H), 4.09 (br. s, 1H), 4.02 (br. d, *J* = 8.6, 1H), 3.97–3.70 (m, 6H), 3.33 (br. s, 2H), 2.80 (br. s, 1H), 2.65 (br. d, *J* = 6.5, 1H), 2.25 (td, *J* = 7.5, 3.6, 2H), 2.09–1.86 (m, 2H), 1.66–1.58 (m, 2H), 1.58–1.51 (m, 2H), 1.42–1.16 (m, 66H), 0.89 (t, *J* = 7.0, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8, 100.4, 70.8, 70.3, 70.2, 69.3, 67.1, 62.9, 49.3 (dd, *J* = 27, 21), 36.6, 31.9, 30.5 (t, *J* = 23), 30.2–28.3 (31C), 25.5, 22.7, 20.3, 14.1 (2C) [loss of 2C related to fluorine due to bad relaxation]. ^{19}F NMR (376 MHz, $CDCl_3$) δ -113.0 (ddt, *J* = 267, 26, 9, 1F), -114.5 (m, 1F), -117.2 (d, *J* = 275, 1F), -119.7 (d, *J* = 275, 1F). MS (MALDI+) m/z 920.7 $[M + Na]^+$. HRMS (MALDI+) for $C_{50}H_{95}F_4NNaO_7^+$ $[M + Na]^+$ calcd. 920.6937, found 920.6931. Elemental Analysis calcd. C, 65.86; H, 10.67; N, 1.54 (**11**+0.75H_2O); found C, 65.92; H, 10.65; N, 1.45.

5. Experimental for biological evaluation:

***In vitro* assays for human iNKT cell stimulation**: Human 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 anti-mouse IgG-coated magnetic beads (Dynal, Invitrogen Corp. Carlsbad, CA) and

cultivated in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin (Gibco BRL, Carlsbad, CA) and 300 U/mL IL-2 (Chiron Corp. Emerville, CA). The iNKT cell line that was used (MAD11) contained >90% Vα24/Jα18 positive cells. Human CD1d-transfected HeLa cells were obtained from M. Kronenberg (La Jolla, CA). These antigen-presenting cells were cultivated in DMEM or RPMI 1640, respectively, containing 1 g/L glucose, supplemented as described above. Antigen-presenting cells HeLa-CD1d were plated at 30.000 per well, on 96-well flat bottom plates in complete RPMI and incubated overnight at 37°C with varying concentrations of glycolipids solubilized in DMSO. Synthetic KRN7000 was used as reference in all experiments. The cells were then washed twice with RPMI. Fifteen thousand iNKT cells per well in 200 µL complete RPMI without IL-2 were then added for 6h at 37 °C for the IFN-γ secretion analyses or 24h for the IL-13 secretion analysis. Cell-free supernatants were collected and tested for the presence of either IFN-γ or IL-13 by ELISA (eBiosciences). No glycolipid was added in control wells. Dependency on CD1d was tested using untransfected HeLa cells devoid of CD1d as negative control presenting cells

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Appendix A. Supporting data.

Supplementary data to this article can be found on line at …..

ABREVIATIONS:

AIBN, azobisisobutyronitrile ; AA, amino acids (Asp, aspartic acid; Arg, arginine; Thr, threonine; Ser, Serine ; Phe, phenylalanine ; Gly, glycine); APCs, antigen presenting cells; Boc, *tert*-butyloxycarbonyl; CD, cluster of differentiation (hCD1d, human CD1d; mCD1d, mouse CD1d); DCs, dendritic *cells*; DFT, density functional theory; DMAP, *N*,*N*-dimethylaminopyridine; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-

linked immunosorbent assay ; FCS, Fetal Calf Serum; α-GalCer and KRN7000, α-galactosylceramide; *gem*, germinal; HOMO, highest occupied molecular orbital; IFN-γ, interferon γ; IL, interleukin; *i*NKT, invariant natural killer T; PD-1 Programmed cell death 1; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate ; *QM,* quantum mechanics; RPMI, Roswell Park Memorial Institute medium; TBAF, tetra-n-butylammonium fluoride ; TBDMSCl, tert-butyldimethylsilyl chloride; TCDI, thiocarbonyldiimidazole ; TCR, T cell receptor; T_H, T helper; TNF-α, tumor necrosis factor α;

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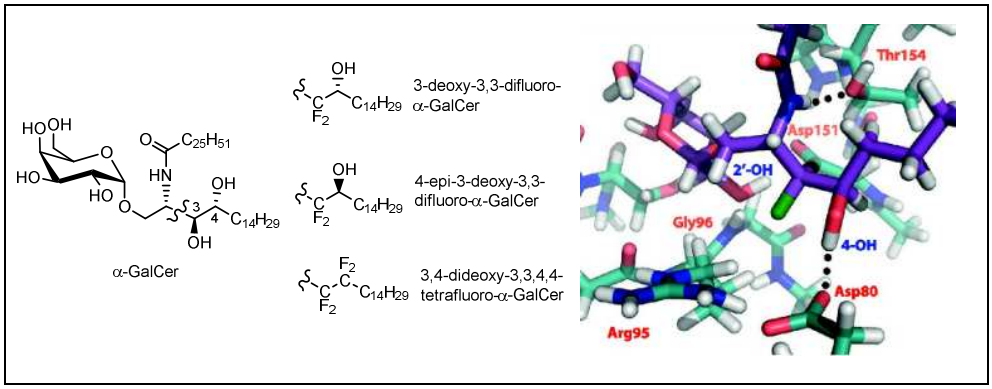
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Graphical abstract



Highlights

- H-bonding of the 3-OH and the amide NH groups on the iNKT stimulation process.
- Synthesis of 3,4-dideoxy-3-fluoro- and 3,4-dideoxy-3,3-difluoro-KRN7000 analogues
- Co-participation of 4-OH on key 3-OH contribution in KRN7000 immune stimulation
- Potency of the tetrafluorinated analogue to highlight contribution of the NH group

Graphical abstract

