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# The Synthesis and in vivo Evaluation of 2′,2′-Difluoro KRN7000

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There has been significant excitement in recent years about the potential of certain glycolipids as immunomodulating agents.[1,2] The discovery of KRN7000 (**1**)[3] can be considered a milestone in this regard (Figure 1). KRN7000 is an exogenous

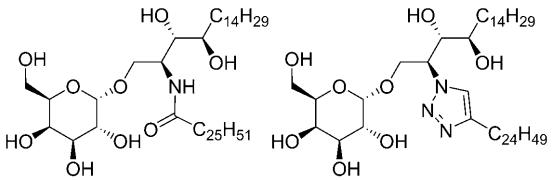


**Figure 1.** Structures of KRN7000 and an amide isostere analogue.[7]

(α-linked) galactosylceramide, and it was shown that its action as an antigen arises by binding to a CD1d protein, followed by recognition of the complex by the semi-invariant T cell receptor (TCR) of natural killer T cells (iNKT cells).[4] The resulting activation of NKT cells leads to a rapid release of both pro-inflammatory Th1 (e.g. IFN-γ and IL-2) and anti-inflammatory Th2 (e.g. IL-4 and IL-10) cytokines.[5]

Unfortunately, the release of both types of cytokines limits the therapeutic potential of **1**, as they antagonise each other's action. Disruption of the carefully controlled Th1/Th2 cytokine balance can cause disease,[6] and therapeutic strategies aimed at a restoration of this balance by in vivo modulation of iNKT cells involving CD1d-restricted antigens that induce a more polarised cytokine response, could be very promising.[2]

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Many analogues of KRN7000 modified at the various substructures have been synthesised and evaluated in murine (m) and human (h) systems. For example, it has been demonstrated that a shorter acyl chain (m,h),[8] as well as introduction of unsaturations (m),[9] results in Th2 polarisation. A shorter phytosphingosine chain gives a preferential Th2 response,[8,10] even when a terminal ring is introduced in a truncated sphingosine chain (h).[11,12a] Introduction of aromatic groups in the acyl chain generally results in a Th1 bias (m,h).[12] Modifications in the galactose unit proved the essential role of the α-anomeric configuration (m),[4,13] and the 2-OH for activity (m,h),[14] but revealed that modifications on the 3-, 4-, or 6-OH groups are allowed (m).[4,14d,15] Interestingly, nonglycosidic (polyhydroxyalkyl) substituted ceramides have been shown to bind to CD1d and activate iNKT cells.[16] The carbocyclic analogue of KRN7000 induces Th1-biased cytokine production (m),[17] as is the case for the corresponding *C*-glycoside (α-*C*-GalCer) (m),[18] and the phytosphingosine C1-nor-*C*-glycoside (m,h).[19] Surprisingly, the corresponding α-*S*-GalCer analogue[20] does not stimulate iNKT cells either in vitro or in vivo.[20b] Analogues with modifications in the phytosphingosine polar region have also been investigated. The 3-OH group proved to be more important than the 4-OH group for antigenic activity (m),[3,21] and the stereochemistry of the alcohols and amide substituents influence the activity (m).[22] Other modifications in this region include conformationally restricted analogues featuring an azetidine ring (in which the amide nitrogen and C4 are linked), which show potent induction of cytokines with a slight Th2 bias (m),[23] and a fluorinated analogue containing a 4-deoxy-4,4-difluoro modification, which shows preferential Th1 induction (m).[24] Finally, Kim and co-workers reported a set of analogues in which the amide group is replaced by a triazole moiety, with variable lengths of the attached lipid chain (m).[7] It was found that selected analogues elicit a Th2-polarised cytokine response, with the long-chained analogues such as **2** having a stronger response.

However, at present the relationship between glycolipid structure and cytokine polarisation is not completely understood. It has been postulated that the origin of cytokine polarisation could relate to the stability of the glycolipid complex with CD1d,[2,8b,12a,25] or to a difference in glycolipid-presenting cells.[26]

The matter is complicated given the two binding events involved. X-ray crystallographic studies have given some insight into these binding events. The crystal structure of human CD1d in complex with KRN7000 shows how the long aliphatic chains fit in 'binding grooves' present on CD1d.[27] Importantly, it was also discovered that a series of hydrogen bonds between the galactosylceramide and CD1d serve to position the

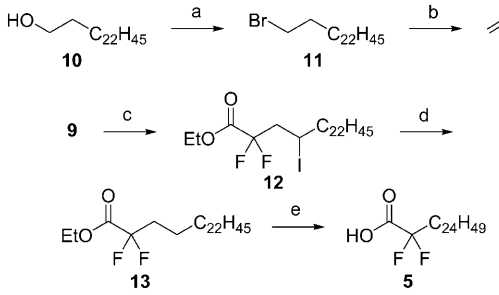
  

galactose ring above the CD1d surface. Important hydrogen bonds occur between hCD1d Asp151 (equivalent to mCD1d Asp153) and the galactose 2-OH group, and between Asp80 and the phytosphingosine 3-OH group. A hydrogen bond between Thr154 and the α-galactosyl acetal oxygen atom was also suggested, but this was not supported by molecular dynamics simulations.[28] X-ray crystallographic analysis of mCD1d in complex with a short-chain variant of KRN7000 revealed that Asp80 is hydrogen bonded to both the sphingosine 3-OH and 4-OH groups, and that Asp153 interacts with both the galactose 2-OH and 3-OH groups.[26a] Interestingly, a hydrogen bond between the OH group of Thr156 and the KRN7000 amide NH group was indicated, with no direct involvement of the amide oxygen atom in hydrogen bonding with CD1d. A very similar hydrogen bonding pattern emerged from X-ray crystallographic studies of α-galacturonosylceramide (GalA-GSL) complexed to mCD1d (though GalA-GSL lacks a sphingosine 4-OH group).[29] The crystal structure of the KRN7000–CD1d–NKT TCR was solved in 2007,[30] and shows a hydrogen bond between the sphingosine 3-OH and Arg95 of the CDR3α loop of the TCR. Interestingly, the hydrogen bonds mentioned above between KRN7000 and CD1d, involving 3-OH···Asp80 and the galactose 2′′-OH···Asp151, are maintained upon binding with NKT TCR.

We were intrigued by the Thr156–amide NH hydrogen bond. Inspection of the various X-ray structures indeed revealed that the amide N–H is perfectly lined up to form a hydrogen bond with the OH group of the adjacent Thr residue (Thr156 for mCD1d, Thr154 for hCD1d), with the distance between these groups short enough to suggest an interaction. It was decided to investigate the functional role of this possible interaction by altering the amide N–H hydrogen bond characteristics. We therefore introduced two fluorine atoms in the α-position of the amide carbonyl group, leading to **3** as target molecule (Scheme 1). Fluorination of amides in the α-position increases N–H acidity,[31] and hence a stronger interaction was expected if this bond indeed plays an important role in the GalCer–CD1d interaction network. Based on the model regarding the relationship between complex stability and cytokine polarisation mentioned above,[2] a higher complex stability would induce Th1 cytokine polarisation.

The synthesis of **3** was envisioned as shown in Scheme 1, and follows a conventional synthetic strategy to α-galactosylceramides. Disconnection of the amide acyl chain leads to **4**, which is obtained by α-selective glycosylation of the phytosphingosine derivative **7**[32] with the galactosyl trichloroacetimidate donor **6**.[33] The synthesis of the fluorinated fatty acid **5** was envisioned by a fluorinated building block approach involving addition of **8** to alkene **9**.

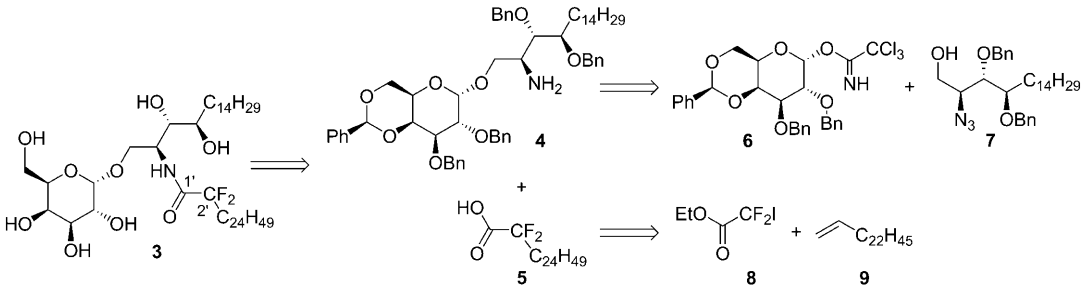
Alkene **9** was synthesised in two steps from commercially available tetracosanol **10** by bromination[34] followed by elimination (Scheme 2). The elimination was achieved using a combination of KOH and a substoichiometric amount of triisopropylsilanol (TIPSOH) in *N*,*N*-dimethylformamide (DMF) at room temperature[35] which furnished the desired alkene in 66% yield, together with 16% of the corresponding nucleophilic substitution product ($TIPSOC_{24}H_{49}$). By using a mixed solvent system (DMF/$Et_2O$ 85:15), an improved yield (73%) of the alkene **9** was obtained, together with 21% yield of the silyl ether byproduct. AIBN-mediated radical addition of **8**[36] gave the addition product **12** in excellent yield. Subsequent iodide reduction with $Bu_3SnH$ using room temperature initiation with $Et_3B$ gave **13** in good yield, together with 16% of starting material. Finally, ester saponification proceeded in almost quantitative yield to give the desired difluorinated carboxylic acid **5**.




**Scheme 2.** Synthesis of the fluorinated fatty acid **5**: a) DDQ, TBAB, $PPh_3$, $CH_2Cl_2$, room temperature, 30 min, 96%; b) KOH, TIPSOH, DMF/$Et_2O$ (85:15), room temperature, 16 h, 73%; c) **8**, AIBN, DCE, 70 °C, 16 h, 87%; d) $Bu_3SnH$, $Et_3B$, air, toluene, room temperature, 1 h, 70%; e) NaOH (1 M), THF, room temperature, 16 h, 95%.

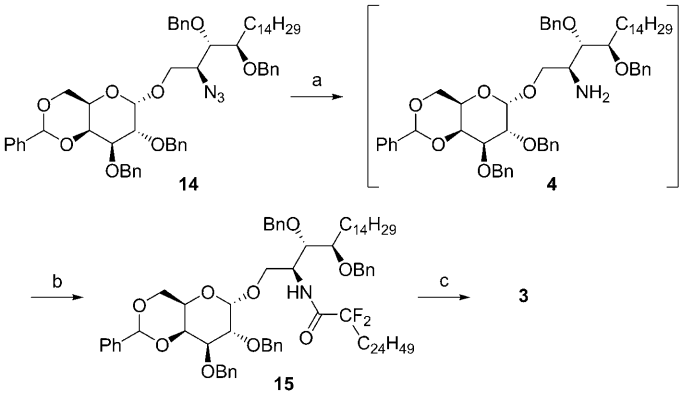
A direct fluorination approach towards **5** involving LDA-mediated deprotonation of commercially available $MeOOCC_{25}H_{51}$ followed by reaction with NFSI[37] was not successful, as the formation of Claisen self-condensation byproduct was always observed, even at −78 °C (not shown).

The final sequence to the αGalCer analogue **3** is given in Scheme 3. The α-galactosylphytosphingosine derivative **14** was synthesised by glycosylation of **7** with **6** according to pub-




**Scheme 1.** Retrosynthetic analysis for compound **3**.




**Scheme 3.** Synthesis of the fluorinated KRN7000 **3**: a) $PMe_3$, THF, 0 °C→room temperature, NaOH (1 M); b) **5**, EDC, $CH_2Cl_2$, 41%; c) $H_2$/Pd black, $CHCl_3$/EtOH, 50%.

lished methods.[38] Azide reduction gave rise to the amine **4**, which was, without purification, submitted to EDC-mediated amide bond formation[33b] with **5** to give **15** in reasonable yield. Given the increased electrophilicity of the ester moiety in **13**, we attempted to react **13** directly with the amine in **4** according to literature precedent,[39] but this proved unsuccessful. Finally, global deprotection led to the desired target **3**.

To investigate whether compound **3** could induce a biased immune response compared with compound **1**, the serum cytokine levels were measured after injection of both glycolipids into C57Bl/6 mice (Figure 2). The data demonstrate that similar levels of IL-4 were induced, while a decrease in IFN-γ production was detected after injection of compound **3** relative to compound **1**. No cytokine induction was observed when compound **3** was injected in either J$\alpha$18$^{-/-}$ or CD1d$^{-/-}$ mice, indicating that this glycolipid induced CD1d-dependent TCR activation of NKT cells.

These results indicate that fluorination at the 2-position in the acyl chain influences cytokine polarisation, with a comparable magnitude of NKT cell response. Based on the hydrogen bond argument advanced above, this is an unexpected outcome. However, other effects involving fluorine substitution that influence the biological response cannot be excluded. For example, there could be a stronger solvation of the fluorinated amide moiety, leading to a higher penalty for desolvation of the amide, which could compensate for a stronger hydrogen bond. We do not expect a significant steric effect because Koezuka and co-workers have reported that the 2′-OH group has no significant effect in their assays,[3] and the $CF_2$ group is a good steric match for a CHOH group.[40] There could be conformational effects involved. The crystal structures reveal that the carbonyl oxygen of KRN7000 almost eclipses the acyl chain (small O=C1–C2–C3 torsion angle). O'Hagan and co-workers established that in the α-fluoroamide F–C–C=O moiety, the C–F bond preferentially adopts a conformation antiperiplanar to the C=O bond (C3–C2–C1=O torsion angle: 60°), and the potential energy minimum was calculated to be up to 8 kcal mol$^{-1}$.[41] However, calculations by Seebach and co-workers for a geminal difluorinated amide derivative suggest a more shallow torsional potential, where all maxima and minima were found within 3 kcal mol$^{-1}$ from the lowest-energy conformer, which also had a C–F bond antiperiplanar to the C=O bond.[42] From these data, it can be concluded that the fluorination in **3** is likely to have only a small destabilising conformational effect if the same binding mode as that of KRN7000 is adopted. Hence, the direction of cytokine polarisation could either suggest that the amide NH···Thr156 hydrogen bond, if indeed stronger, plays no major role in stabilising the CD1d–GalCer interaction, or, following Hénon's assertion that the amide is involved in a hydrogen bond network responsible for the orientation of the polar head group,[28] the change in that network resulting from fluorine introduction—possibly as a result of the conformational effects—could negatively impact the polar group stabilisation, consistent with a lower affinity with the TCR. In that sense, our results are in line with data reported by Lee et al., involving the amide analogue **2**,[7] in which a change in the hydrogen bonding network between the CD1d–**2**–TCR leads to an observed Th2 polarisation as well.

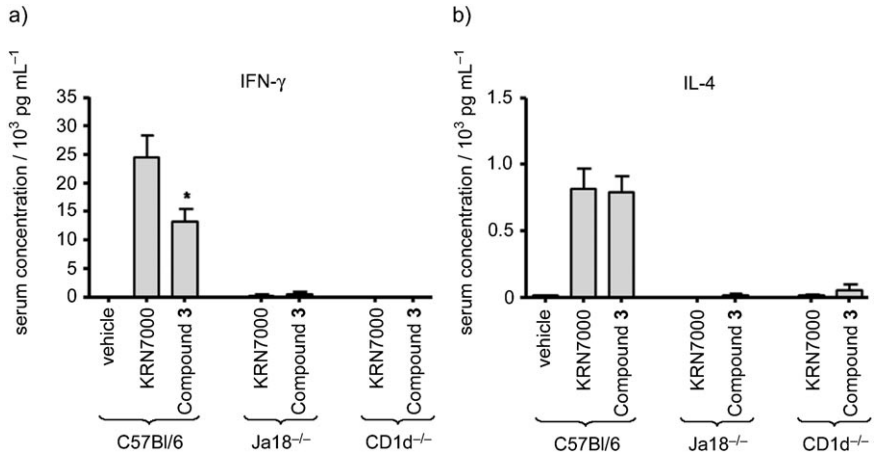



**Figure 2.** Fluorination of KRN7000 elicits CD1d-dependent TCR-activation of NKT cells: C57Bl/6, CD1d$^{-/-}$, or J$\alpha$18$^{-/-}$ mice were injected with the indicated compounds or vehicle, and serum cytokine levels were assayed for a) IFN-γ and b) IL-4 by ELISA. Data given are the mean of 5–9 mice per group ± SEM. (*$P < 0.05$ vs. KRN7000).

In conclusion, we have successfully synthesised a novel KRN7000 analogue that has the potential to interrogate molecular interactions at the atomic level. Our biological data show that compound **3** is able to modulate NKT cell responses and that the presence of two fluorine atoms in the α-position of the amide group induces a Th2-biased immune response by murine NKT cells.

## Experimental Section

Column chromatography was performed on 230–400 mesh Matrex silica gel. Preparative HPLC was

carried out using a Bio-Rad Biosil D 90–10, 250×22 mm column eluting at 20 mL min$^{-1}$. Melting point values are uncorrected. Reaction solvents were dried before use as follows: THF and $Et_2O$ were distilled from sodium/benzophenone; $CH_2Cl_2$ and $Et_3N$ were distilled from $CaH_2$; toluene was distilled from sodium; pyridine was double distilled from $CaH_2$ and stored in a Schlenk flask. Ethyl iododifluoroacetate (light pink) was purchased from Fluorochem Ltd., and in most cases was used without further purification. If the purchased ethyl iododifluoroacetate was deep purple in colour, the reagent was dissolved in $Et_2O$, washed with a saturated solution of $Na_2S_2O_3$, and concentrated in vacuo to give a light-yellow oil. All reaction vessels were flame dried under vacuum prior to use, and all experiments were carried out under $N_2$ atmosphere. All other reagents were purchased from commercial sources and used without further purification.

**1-Bromotetracosane (11)**: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ; 833 mg, 3.01 mmol) was added to a mixture of alcohol tetracosanol **10** (1.0 g, 2.73 mmol), $PPh_3$ (789 mg, 3.01 mmol), and tetra-*n*-butylammonium bromide (TBAB; 970 mg, 3.01 mmol) in $CH_2Cl_2$ (13.6 mL), and the mixture was stirred at room temperature for 30 min. The deep-red mixture was poured into $H_2O$/MeOH (1:1, 100 mL), extracted with petroleum ether (3×80 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude was purified by column chromatography (petroleum ether) to afford bromide **11** as a colourless oil (1.10 g, 96%). $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 3.41 (2H, t, $J$ = 7.0 Hz), 1.87 (2H, quintet, $J$ = 7.0 Hz), 1.49–1.21 (42H, m), 0.90 ppm (3H, t, $J$ = 6.5 Hz); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 33.9, 33.1, 32.1, 29.90–29.82 (m), 29.75, 29.64, 29.56, 29.0, 28.4, 22.9, 14.3 ppm. $^1$H and $^{13}$C NMR correspond to previously reported values.[43]

**Tetracos-1-ene (9)**: KOH (352 mg, 6.27 mmol) was added to a mixture of bromide **11** (1.31 g, 3.13 mmol) and TIPSOH (0.62 mL, 3.13 mmol) in DMF (31 mL) and $Et_2O$ (5 mL), and the mixture was stirred at room temperature for 16 h. The mixture was poured into $H_2O$ (60 mL), extracted with petroleum ether (3×80 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude was purified by column chromatography (petroleum ether) to afford alkene **9** (0.774 g, 73%) and the corresponding nucleophilic substitution product, $TIPSOC_{24}H_{49}$ **9b** (334 mg, 21%), both as colourless oils. Alkene **9** was used immediately to avoid polymerisation. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 5.82 (1H, ddt, $J$ = 17.0, 10.0, 6.5 Hz), 5.00 (1H, m), 4.94 (1H, ddt, $J$ = 10.5, 2.0, 1.5 Hz), 2.08–2.02 (2H, m), 1.39–1.11 (40H, m), 0.91–0.84 ppm (3H, m); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 139.4, 114.2, 34.0, 32.1, 29.9, 29.8 (m), 29.5, 29.3, 29.2, 29.1, 22.9, 14.3 ppm; IR: $\tilde{\nu}_{max}$ = 2924 (m), 2853 (m), 1466 (w), 1264 (m), 738 cm$^{-1}$ (s). Data for **9b**: $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 3.68 (2H, t, $J$ = 6.5 Hz), 1.55 (2H, quintet, $J$ = 7.0 Hz), 1.38–1.20 (42H, m), 1.15–1.01 (21H, m), 0.89 ppm (3H, t, $J$ = 7.0 Hz). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 63.7, 33.3, 32.1, 29.9, 29.82, 29.68, 29.56, 26.0, 22.9, 18.2, 14.3, 12.2 ppm; IR: $\tilde{\nu}_{max}$ = 2918 (vs), 2850 (s), 1466 (w), 1105 cm$^{-1}$ (w); EIMS *m/z* (%): 467 ([*M*−*i*Pr]$^+$, 100); HRMS (EI) for $C_{33}H_{69}OSi$ [*M*−H]$^+$: calcd 509.5118, found 509.5134.

**Ethyl 2,2-difluoro-4-iodohexacosanoate (12)**: 2,2′-azobisisobutyronitrile (AIBN; 37.5 mg, 0.228 mmol) was added to a solution of alkene **9** (770 mg, 2.28 mmol) and iododifluoroacetate **8** (0.41 mL, 2.97 mmol) in 1,2-dichloroethane (DCE; 11.4 mL). The mixture was stirred at 70 °C for 16 h, followed by concentration in vacuo. The crude was purified by column chromatography (EtOAc/petroleum ether 5:95) to afford the difluoroester **12** as a colourless oil (1.17 g, 87%). $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 4.36 (2H, q, $J$ = 7.0 Hz), 4.23 (1H, dtd, $J$ = 8.5, 7.0, 4.5 Hz), 2.92 (1H, dtd, $J$ = 18.5, 16.0, 6.5 Hz), 2.74 (1H, dddd, $J$ = 18.0, 15.5, 12.5, 7.0 Hz), 1.87–1.69 (2H, m), 1.38 (3H, t, $J$ = 7.0 Hz), 1.59–1.21 (40H, m), 0.89 ppm (3H, t, $J$ = 7.0 Hz); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 163.6 (t, $J$ = 32.5 Hz), 115.4 (t, $J$ = 251.0 Hz), 63.4, 45.6 (t, $J$ = 23.0 Hz), 40.7, 32.1, 29.9 (m), 29.8, 29.7, 29.6, 29.5, 28.7, 23.5 (t, $J$ = 3.5 Hz), 22.8, 14.3, 14.1 ppm; $^{19}$F NMR (282 MHz, $CDCl_3$): $\delta$ = −102.3 (d, $J$ = 262.5 Hz), −107.0 ppm (d, $J$ = 264.5 Hz); IR: $\tilde{\nu}_{max}$ = 2916 (s), 2849 (s), 1770 (w), 1467 cm$^{-1}$ (w); ES$^+$ MS *m/z* (%): 609 ([*M*+Na]$^+$, 30), 413 (100); HRMS (ES$^+$) for $C_{28}H_{53}F_2IO_2Na$ [*M*+Na]$^+$: calcd 604.3397, found 604.3388.

**Ethyl 2,2-difluorohexacosanoate (13)**: $Et_3B$ (17 μL, 0.017 mmol) was added to a solution of $Bu_3SnH$ (29 μL, 0.105 mmol) and iododifluoroacetate **12** (51.3 mg, 0.0875 mmol) in toluene (0.87 mL). The mixture was stirred at room temperature for 16 h and purified by column chromatography ($CH_2Cl_2$/petroleum ether 1:4) to afford iododifluoroester **13** (14.6 mg, 36%) as a waxy solid. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 4.33 (2H, q, $J$ = 7.0 Hz), 2.12–1.99 (2H, m), 1.55–1.41 (2H, m), 1.36 (3H, t, $J$ = 7.0 Hz), 1.38–1.21 (42H, m), 0.89 ppm (3H, t, $J$ = 7.0 Hz); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 164.6 (t, $J$ = 32.0 Hz), 116.5 (t, $J$ = 248.0 Hz), 62.8, 34.7 (t, $J$ = 22.5 Hz), 32.1, 29.9 (m), 29.8, 29.7, 29.6, 29.5, 29.4, 29.2, 22.8, 21.6 (t, $J$ = 4.5 Hz), 14.2, 14.1 ppm; $^{19}$F NMR (282 MHz, $CDCl_3$): $\delta$ = −106.1 ppm; IR: $\tilde{\nu}_{max}$ = 2916 (vs), 2849 (vs), 1761 (m), 1465 (m), 735 cm$^{-1}$ (m); ES$^+$ MS *m/z* (%): 483 ([*M*+Na]$^+$, 100); HRMS (ES$^+$) for $C_{28}H_{54}F_2O_2Na$ [*M*+Na]$^+$: calcd 483.3984, found 483.3984.

**2,2-Difluorohexacosanoic acid (5)**: NaOH (1 M, 1 mL) was added to a solution of **13** (27.6 mg, 0.060 mmol) in THF (1 mL). The mixture was stirred at room temperature for 16 h, followed by the addition of HCl (2 M, 1.5 mL). The aqueous layer was extracted with $Et_2O$ (3×10 mL), the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude was used immediately in the subsequent transformation.

**Protected 2′,2′-difluoro KRN7000 15**: 1) $PMe_3$ (1.0 M solution in THF; 1.0 mL, 1.0 mmol) was added to azide **14** (200 mg, 0.210 mmol) in THF at 0 °C, and the mixture was stirred at 0 °C for 45 min, followed by a further 3 h at room temperature. NaOH (1 M, 2 mL) was added, and the mixture was stirred for 80 min at room temperature. EtOAc (10 mL) was then added, and the organic layer was washed with $H_2O$ (2×10 mL) and brine (10 mL), dried over $Na_2SO_4$, filtered, and concentrated in vacuo to give crude amine **4** (197 mg). The crude (61 mg) was used immediately in the subsequent transformation. 2) EDC (22 mg, 0.141 mmol) was added to a mixture of crude amine **4** (61 mg, 0.0658 mmol) and difluorohexacosanoic acid **5** (20 mg, 0.0462 mmol) in $CH_2Cl_2$ (2 mL), and the mixture was stirred at room temperature for 17 h. $CH_2Cl_2$ (5 mL) was added, and the solution was washed with $H_2O$ (10 mL) and brine (10 mL), dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The crude was purified by column chromatography (EtOAc/hexane 1:9) to afford the protected α-GalCer **15** as a solid (25 mg, 40%); mp: 56–58 °C; $[\alpha]_D^{29}$ = +46.0 ($c$ = 0.125, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ = 7.53–7.50 (2H, m), 7.42–7.22 (23H, m), 6.83 (1H, d, $J$ = 8.5 Hz), 5.45 (1H, s), 4.91 (1H, d, $J$ = 3.5 Hz), 4.85 (1H, d, $J$ = 11.8 Hz), 4.78 (1H, d, $J$ = 12.0 Hz), 4.72 (1H, d, $J$ = 12.0 Hz), 4.71 (1H, d, $J$ = 11.5 Hz), 4.64 (1H, d, $J$ = 11.8 Hz), 4.56 (1H, d, $J$ = 11.6 Hz), 4.51 (1H, d, $J$ = 11.3 Hz), 4.49 (1H, d, $J$ = 11.6 Hz), 4.33 (1H, m), 41.6–4.05 (3H, m), 3.95–3.88 (3H, m), 3.83–3.76 (2H, m), 3.53–3.50 (2H, m), 2.07–1.95 (2H, m), 1.71–1.56 (2H, m), 1.45–1.37 (2H, m), 1.26 (m, br), 0.89 ppm (6H, appt, $J$ = 6.8 Hz); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 138.64, 138.59, 138.3, 138.0, 137.8, 128.8, 128.5, 128.4, 128.3, 128.1, 127.8 (m), 127.7, 127.60, 127.57, 126.3, 100.0, 99.6, 79.5, 79.2, 76.2, 75.5, 74.4, 73.8, 73.5, 72.0, 71.9, 69.3, 67.6, 63.1, 50.6, 33.8 (t, $J$ = 23.0 Hz), 31.9, 30.2, 29.7 (m), 29.6, 29.4, 29.2, 25.8, 22.7, 21.6 (m), 14.1 ppm (amide and $CF_2$ not observed); $^{19}$F NMR (282 MHz, $CDCl_3$): $\delta$ = −105.4 (d, $J$ = 252.5 Hz), −107.2 ppm

(d, $J=252.5$ Hz); IR: $\tilde{\nu}_{max}=3319$ (w, br), 2918 (s), 2850 (m), 1674 (m), 1549 (m), 1496 (w), 1453 (w), 1467 (w), 1097 (s), 1048 (s), 724 (s), 695 $cm^{-1}$ (s); $ES^+MS$ *m/z* (%): 1360 ($[M+NH_4]^+$, 20), 1365 ($[M+Na]^+$, 100).

**2′,2′-Difluoro KRN7000 (3)**: Pd black (6 mg, 0.05 mmol) was added to a mixture of protected α-GalCer **15** (23 mg, 0.017 mmol) in EtOH/$CHCl_3$ (3:1, 1 mL) and the mixture was stirred at room temperature under $H_2$ atmosphere (balloon) for 1.5 h. Pyridine (~ 0.2 mL) was added, and the mixture was stirred for 15 min at room temperature, filtered through celite, washed with a copious amount of $CH_2Cl_2$/MeOH (1:1) and co-evaporated with toluene under reduced pressure. The crude was purified by column chromatography (MeOH/$CH_2Cl_2$ 10, 20, then 50% *v/v*) to afford α-GalCer **3** as a solid (7 mg, 46%). $^1H$ NMR (400 MHz, $CDCl_3/CD_3OD$): $\delta=4.85$ (1H, d, $J=4.0$ Hz), 4.18 (1H, m), 3.89 (1H, d, $J=3.5$ Hz), 3.86 (1H, dd, $J=10.5$, 4.5 Hz), 3.73 (1H, m), 3.70–3.67 (2H, m), 3.66–3.61 (2H, m), 3.53–3.48 (2H, m), 3.33–3.27 (2H, m), 2.07–1.87 (2H, m), 1.57–1.06 (m), 0.86–0.72 ppm (6H, apt, $J=5.7$ Hz); $^{19}F$ NMR (282 MHz, $CDCl_3/CD_3OD$): $\delta=-105.7$ (d, $J=254.0$ Hz), −108.0 ppm (d, $J=251.5$ Hz); IR: $\tilde{\nu}_{max}=3292$ (m, br), 2916 (s), 2849 (s), 1675 (m), 1546 (w), 1467 (m), 1029 $cm^{-1}$ (m); $ES^+MS$ *m/z* (%): 916 ($[M+Na]^+$, 100).

**In vivo stimulation with glycolipids:** C57Bl/6J (B6) mice were originally purchased from The Jackson Laboratory, while both $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice (both on B6 background) were kindly provided by Dr. François Trottein (Lille, France). Mice were bred in our breeding facility, and treated and used in agreement with the institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Ethics Committee. Stock solutions of α-GalCer and compound **3** were prepared in Tween-20/So-deoxycholate/sucrose, which are further referred to as vehicle. Before use, the solutions were diluted with phosphate-buffered saline (pH 7.4) to obtain a final concentration of 10 μg $mL^{-1}$. Mice were injected intraperitoneally with 5 μg of glycolipid or with diluted vehicle alone. Sera were collected at two time points, and the levels of both IFN-γ (at 16 h) and IL-4 (at 2 h) were measured by a standard sandwich ELISA using purified capture and biotin-conjugated detection monoclonal antibodies and standards. After incubation with avidin–peroxidase, ELISAs were developed with TMB substrate, followed by evaluation using a microplate reader.

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