

Synthesis and Evaluation of *N*-Acetyl-2-amino-2-deoxy- α -D-galactosyl 1-Thio-7-oxaceramide, a New Analogue of α -D-Galactosyl Ceramide

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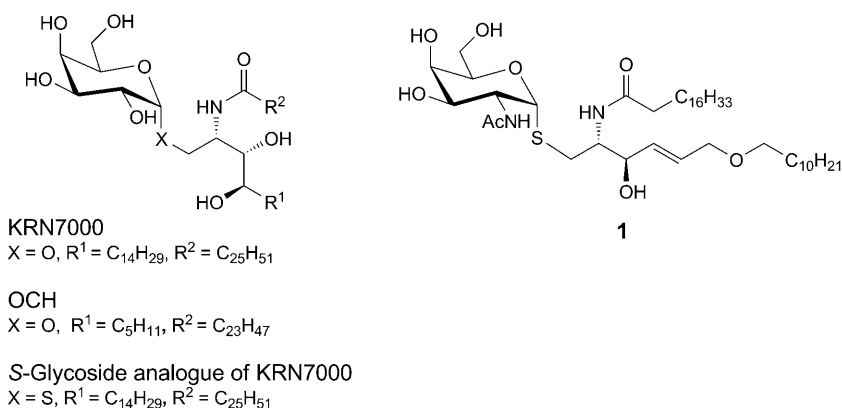
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The *N*-acetyl-2-amino-2-deoxy- α -D-galactopyranosyl 1-thio-7-oxaceramide **1** was synthesized by substituting the 7-oxasphingosine triflate **3** with α -D-*N*-acetyl-1-thiogalactosamine (**2**). The triflate **3** was obtained from azide **4**. Thiol **2** was prepared according to a known procedure from α -D-galactosamine hydrochloride. As compared to ceramide (Cer), **1** is neither a substrate of ceramide kinase (CerK), consistent with the absence of the C(1)–OH group, nor an inhibitor of Cer phosphorylation by CerK. While **1** partially displaced CD1d-bound lipids, it failed to stimulate invariant natural killer T (iNKT) cells when presented by human CD1d-transfected cells. These results suggest that **1** binds weakly to recombinant CD1d, but does not form immunogenic complexes with CD1d.

Introduction. – Several α -D-galactopyranosyl ceramides [1–5], especially KRN-7000 [6][7], OCH [8], and RCAI-61¹⁾ [9], incorporating phytosphingosine or sphingosine moieties, possess important biological properties [10–13], comprising immunostimulating [14–16] and antitumor activities [17–24]. Several analogues of these α -D-galactopyranosides were synthesized, and they also display notable biological activities [8][14][25–37]. The synthesis and evaluation of a thioglycoside analogue of KRN7000 has recently been published [38], prompting us to report our results on the synthesis and evaluation of *N*-acetyl-2-amino-2-deoxy- α -D-galactopyranosyl 1-thio-7-oxaceramide **1**. Replacing the galactosyl by an *N*-acetyl-2-amino-2-deoxygalactosyl moiety was thought to contribute to elucidating the effect of C(2)–OH of the glycon on T-cell receptor (TCR) recognition, while the S–glycosyl bond will confer stability against chemical and enzymatic cleavage [39–42]. 7-Oxasphingosines behave very similarly to ceramides, and allow to independently modify the head group and lipid moiety of sphingosine, an advantage that has since been realized using olefin metathesis [43][44].

Conceivably, **1** may be obtained either by glycosidation [45] of a 1-thioceramide or of a 1-thiosphingosine, or by nucleophilic substitution by an *N*-acetyl α -D-galactopyranosyl thiol of a ceramide possessing a leaving group at C(1) and preferentially a non-participating neighbouring group, such as an N₃ substituent [46][47]. We opted for the

¹⁾ OCH is a truncated and RCAI-61 the 6'-*O*-methyl analogue of KRN7000.

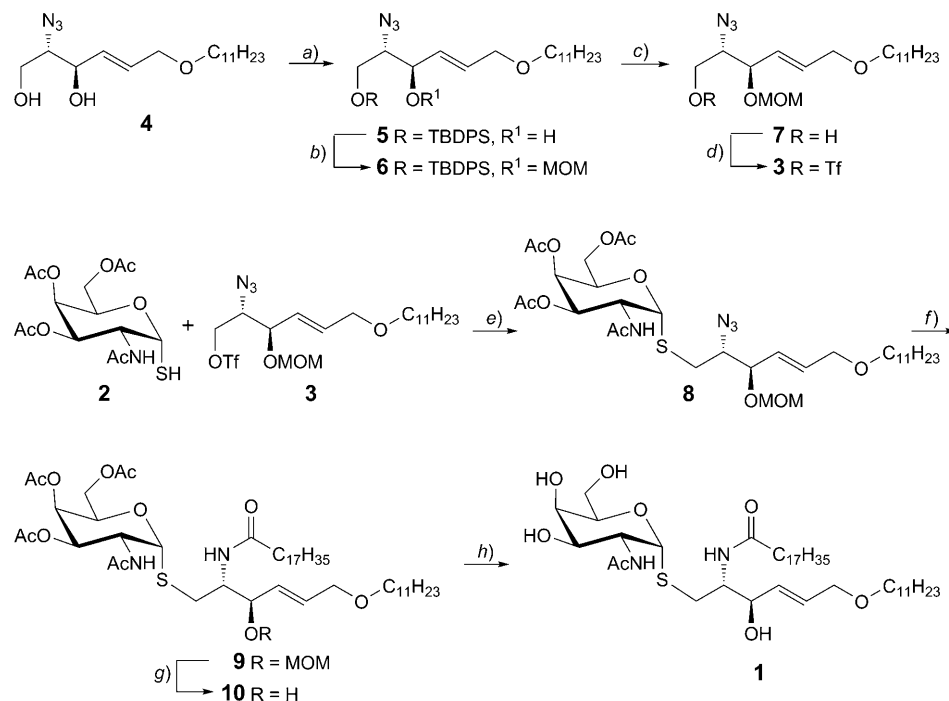


second strategy, assuming that nucleophilic substitution will prevail over single electron transfer from the thiolate to the N_3 group, and report on the synthesis of **1** and the results of its evaluation as substrate of Cer kinase [48] and as T cell-stimulatory antigen [49].

Synthesis. – The synthesis of the *N*-acetyl-2-amino-2-deoxy- α -D-galactopyranosyl 1-thio-7-oxaceramide **1** is shown in the *Scheme*. The thiogalactoside **8** was obtained in 75% yield by the reaction between 2-acetamido-2-deoxy- α -D-galactopyranosyl thiol (**2**) [50] and the 7-oxasphingosine azido triflate (=trifluoromethylsulfonate) **3**. The thiol **2** was synthesized in three steps and in an overall yield of 99% from 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy- α -D-galactopyranose, according to the procedure of *Knapp* and *Myers* [50]. The azido triflate **3** was prepared from azide **4**, derived from 7-oxasphingosine [51], by first silylating the hydroxymethyl group with $t\text{BuPh}_2\text{SiCl}$ (TBDPSCI) to provide **5** in 81% yield and then protecting the HO–C(3) group by treating it with MeOCH_2Cl (MOMCl) in the presence of *Hünig's* base, to yield 91% of **6**. The alcohol **7** was obtained in a yield of 95% by desilylating **6**, and converted to the triflate **3**. The triflate, obtained as a pale yellow oil, was directly subjected to substitution by the thiol **2** in the presence of *Hünig's* base, to provide 75% of the protected *S*-galactosaminide **8** ($J(1,2) = 5.4$). Reducing this galactosaminide by the action of polymer-bound Ph_3P [52], followed by *N*-acylation with stearic anhydride, yielded 86% of the protected ceramide **9** that was deprotected. The MOM group was removed by treating **9** with Tf_2O in MeCN to provide **10** in 87% yield [53][54]. Deacetylation of **10** was effected with 2M NH_3 in MeOH to yield 96% of the desired *N*-acetyl- α -D-galactopyranosyl 1-thio-7-oxaceramide **1**. The α -D configuration is evidenced by $J(1,2) = 5.4$ Hz.

Biological Studies. – 1. *Phosphorylation by Ceramide Kinase* (CerK). Phosphorylation of **1** by ceramide kinase (CerK) was assayed using an established *in vitro* radioassay [55]. As compared with C8-ceramide, **1** was not phosphorylated by CerK (*Fig. 1, a*), in agreement with previous observations showing the requirement for a free OH group on the first C-atom of ceramide to allow for phosphorylation by CerK [56].

Scheme



a) $\text{t-BuPh}_2\text{SiCl}$ (TBDPSCl), 1*H*-imidazole, 4-(dimethylamino)pyridine (DMAP), CH_2Cl_2 ; 81%. b) MeOCH_2Cl (MOMCl), *Hünig's* base, CH_2Cl_2 ; 91%. c) $\text{Bu}_4\text{NF} \cdot 3 \text{H}_2\text{O}$, AcOH, THF; 95%. d) Tf_2O , py, CH_2Cl_2 . e) *Hünig's* base, CH_2Cl_2 ; 75%. f) **1**. Polymer-bound PPh_3 , 1,4-dioxane; **2**. stearic anhydride; 86%. g) Tf_2O , MeCN; 87%. h) 2M NH_3 in MeOH; 96%.

In addition, **1** did not inhibit the phosphorylation of ceramide by CerK, suggesting that **1** does not compete with ceramide for binding to CerK (Fig. 1, b), in contrast to the recently identified CerK inhibitor NVP-231 [57].

2. Human CD1d Binding and T-Cell Activation. The galactosaminide **1** is neither cytotoxic to antigen-presenting cells (APC) nor to iNKT cells at the tested dosage (up to 20 $\mu\text{g/ml}$), as assessed by flow cytometry (data not shown).

We first investigated whether **1** binds recombinant human CD1d and displaces the strong agonist α -D-galactosyl ceramide (αGalCer) that activates iNKT cells and induces release of large amounts of cytokines from this cell population. CD1d was attached to the plastic and incubated for 4.5 h with a tenfold molar excess of **1** and then with αGalCer (2 $\mu\text{g/ml}$). Compound **1** partially reduced the activation of iNKT cells (Fig. 2, a), indicating a weak capacity to prevent binding of αGalCer to CD1d under these experimental conditions. Next, we investigated whether **1** activates iNKT cells when presented by living APC. Different APC were used to exclude cell type-specific effects. In all experiments, there was no activation of iNKT cells (Fig. 2, b). Lack of iNKT cell stimulatory activity may be ascribed to the replacement of the OH group at

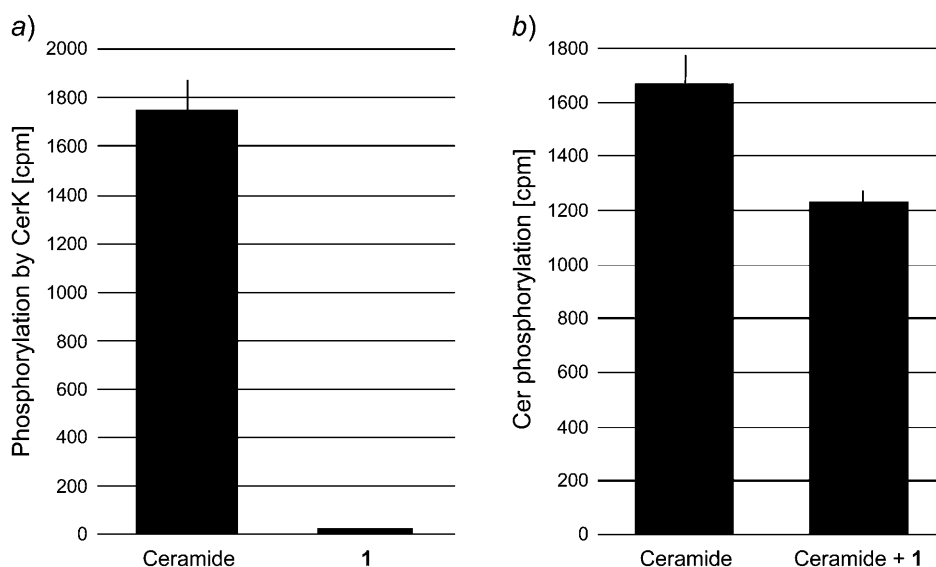


Fig. 1. Phosphorylation of ceramide and the ceramide analogue **1**, assayed as described in the Method section. a) Direct assay showing that **1** is not a substrate for CerK; b) indirect assay showing that **1** does not prevent phosphorylation of ceramide by CerK.

C(2) of the glycon by the acetamido group. Replacements of OH at C(2) of the glycon with an NH₂ group also abolishes iNKT cell activation [7]. The OH group at C(2) establishes H-bonding with two amino acids (R95 and G96) on the alpha chain of iNKT TCR [58] that is mandatory for T-cell activation, as shown by the CD1d: α GalCer binding footprint for the NKT TCR [59]. The observed weak displacement capacity of **1** may be ascribed to the different constitution and rigidity of the oxasphingosine moiety of **1** as compared to the phytosphingosine moiety of α GalCer. The different structure of oxasphingosine might impair the interaction between lipid **1** and the CD1d F' pocket, and influence the position of the sugar head, preventing an optimal interaction with the TCR.

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Experimental Part

1. *Synthesis. General.* THF was distilled from Na and benzophenone, CH₂Cl₂ from P₂O₅, and MeOH and MeCN from CaH₂. Reactions were carried out under Ar, unless stated otherwise. Qual. TLC: precoated silica-gel plates (Merck silica gel 60 F₂₅₄); detection by heating with 'mostain' (400 ml of 10% H₂SO₄ soln., 20 g of (NH₄)₆Mo₇O₂₄·6 H₂O, 0.4 g of Ce(SO₄)₂). Flash chromatography (FC): silica gel Fluka 60 (0.04–0.063 mm). Optical rotations: 1-dm cell at 25°, 589 nm. FT-IR spectra: KBr or ca. 2% soln. in CHCl₃, absorption in cm⁻¹. ¹H- and ¹³C-NMR Spectra: chemical shifts δ in ppm rel. to Me₄Si as external standard, and coupling constants *J* in Hz. HR-MALDI-MS: in gentisic acid (=2,5-dihydroxybenzoic acid, DHB) matrix.

(4E)-2-Azido-1-O-[(tert-butyl)diphenylsilyl]-2,4,5-trideoxy-6-O-undecyl-D-erythro-hex-4-enitol (**5**). A suspension of **4** [16] (570 mg, 1.74 mmol) and 1H-imidazole (154 mg, 2.26 mmol) in dry CH₂Cl₂

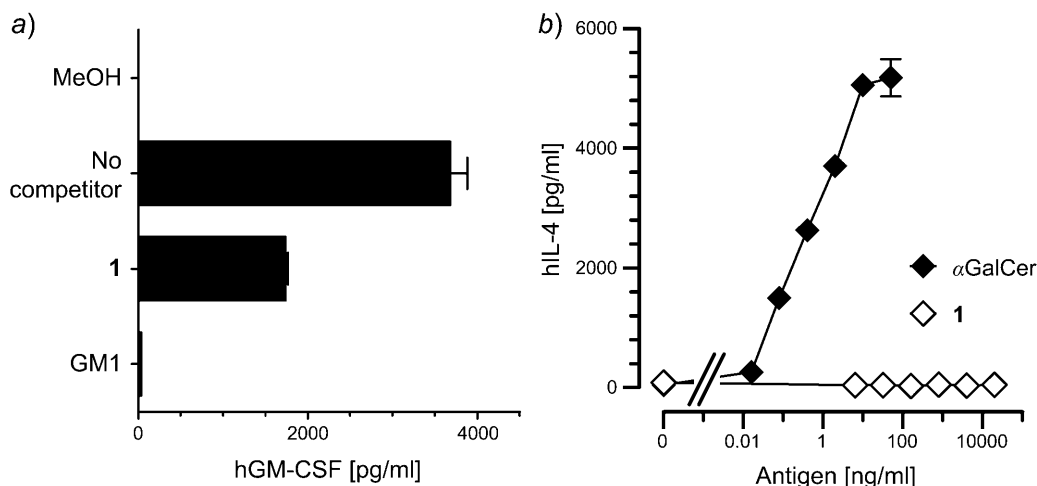


Fig. 2. Binding of **1** to CD1d and failure to activate iNKT cells. *N*-Acetyl- α -D-galactopyranosyl 1-thio-7-oxaceramide (**1**) was tested for its capacity to prevent binding of α GalCer to plate-bound human CD1d and subsequent T-cell response to α GalCer (a). Supernatants were taken after 24 h, and released human GM-CSF and human IL-4 (data not shown) were measured by ELISA and expressed as pg/ml $\pm$ SD of triplicates. Weak but significant competition of **1** with α GalCer was seen as compared to complete inhibition of α GalCer by ganglioside monosialic acid (GM1) used as control competitor. In contrast, **1** ($\diamond$) failed to activate iNKT cells when titrated on CD1d transfectants and compared to α GalCer ($\blacklozenge$) (b). Supernatants were assessed for release of human IL-4, human IFN- γ , and human TNF- α giving similar results.

(10 ml) was treated with t BuPh $_2$ SiCl (TBDPSCl; 0.58 ml, 2.26 mmol) and 4-(dimethylamino)pyridine (DMAP; 21 mg, 0.174 mmol), stirred for 4 h, diluted with H $_2$ O, and extracted with CH $_2$ Cl $_2$ (3 $\times$ 100 ml). The combined org. layers were dried (Na $_2$ SO $_4$) and evaporated. FC (AcOEt/hexane 1:9) gave **5** (797 mg, 81%). Colourless oil. R_f (AcOEt/hexane 1:9) 0.27. $[\alpha]_D^{25} = +13.1$ ($c = 2.0$, CHCl $_3$). IR (CHCl $_3$): 3451w (br.), 3073w, 3012w, 2930s, 2857s, 2105s, 1601w, 1590w, 1471w, 1428w, 1363w, 1264w, 1220m, 1112s, 1007w, 973w, 862w. 1 H-NMR (CDCl $_3$, 300 MHz): 7.70–7.67 (*m*, 4 arom. H); 7.48–7.37 (*m*, 6 arom. H); 5.86 (*dtd*, $J = 15.6, 5.1, 0.9$, H–C(5)); 5.71 (*ddt*, $J = 15.6, 6.3, 1.2$, H–C(4)); 4.30 (*dtd*, $J = 6.3, 5.1, 0.9$, H–C(3)); 3.94 (br. *d*, $J = 5.4$, 2 H–C(6)); 3.85–3.78 (*dd*, $J = 10.8, 6.0$, H–C(1)); 3.80 (*dd*, $J = 10.8, 4.5$, H'–C(1)); 3.51 (*dt*, $J \approx 5.7, 4.5$, H–C(2)); 3.39 (*t*, $J = 6.3$, 2 H–C(1'))); 2.54 (br. *s*, $J = 5.4$, OH); 2.31 (br. *s*, $J = 5.4$, OH); 1.62–1.53 (*m*, 2 H–C(2')); 1.35–1.26 (*m*, 16 H); 1.08 (*s*, Me $_3$ C); 0.88 (*t*, $J = 6.6$, Me). 13 C-NMR (CDCl $_3$, 75 MHz): 135.46 (*4d*); 132.53 (*2s*); 130.54 (*d*, C(4)); 130.08 (*d*, C(5)); 129.82 (*2d*); 127.73 (*4d*); 72.10 (*d*, C(3)); 70.70 (*t*, C(6)); 70.40 (*t*, C(1'))); 66.59 (*d*, C(2)); 64.06 (*t*, C(1)); 31.99, 29.82, 29.70, 29.60, 29.42, 26.26, 22.78 (several *t*); 26.73 (*q*, Me $_3$ C); 19.23 (*s*, Me $_3$ C); 14.32 (*q*, Me). HR-MALDI-MS: 588.3583 (100, $[M + Na]^+$, C $_{33}$ H $_{51}$ N $_3$ NaO $_3$ Si $^+$; calc. 588.3592). Anal. calc. for C $_{33}$ H $_{51}$ N $_3$ O $_3$ Si (565.87): C 70.04, H 9.08, N 7.43; found: C 70.27, H 8.95, N 7.51.

(4E)-2-Azido-1-O-[(*tert*-butyl)diphenylsilyl]-2,4,5-trideoxy-3-O-(methoxymethyl)-6-O-undecyl-D-erythro-hex-4-enitol (**6**). A soln. of **5** (790 mg, 1.39 mmol) and Hünig's base (0.69 ml, 4.19 mmol) in ClCH $_2$ CH $_2$ Cl was treated with MOMCl (0.16 ml, 2.09 mmol), heated to 75°, stirred for 6 h, after which another 0.16 ml of MOMCl was added, stirred further for 16 h at 75°, diluted with H $_2$ O, and extracted with CH $_2$ Cl $_2$ (2 $\times$ 50 ml). The combined org. layers were dried (Na $_2$ SO $_4$) and evaporated. FC (AcOEt/hexane 1:19) gave **6** (773 mg, 91%). Colourless oil. R_f (AcOEt/hexane 1:19) 0.30. $[\alpha]_D^{25} = -36.5$ ($c = 1.7$, CHCl $_3$). IR (CHCl $_3$): 3018m, 2930s, 2857s, 2103m, 1599w, 1471w, 1428w, 1362w, 1217m, 1152m, 1112s, 1005s, 1024m. 1 H-NMR (CDCl $_3$, 300 MHz): 7.70–7.65 (*m*, 4 arom. H); 7.48–7.36 (*m*, 6 arom. H); 5.78 (*dtd*, $J = 15.3, 5.3, 0.6$, H–C(5)); 5.56 (*ddt*, $J = 15.6, 8.1, 1.5$, H–C(4)); 4.65 (*d*, $J = 6.9$, O–CH $_2$ –O); 4.50

(*d*, *J* = 6.9, O–CH₂–O); 4.18 (*dd*, *J* = 7.8, 4.5, H–C(3)); 3.95 (*dd*, *J* = 5.1, 1.2, 2 H–C(6)); 3.77 (*dd*, *J* = 10.5, 5.4, H–C(1)); 3.72 (*dd*, *J* = 10.5, 6.6, H'–C(1)); 3.63 (*dt*, *J* = 6.6, 5.1, H–C(2)); 3.37 (*t*, *J* = 6.6, 2 H–C(1')); 3.28 (*s*, MeO); 1.60–1.50 (*m*, 2 H–C(2')); 1.34–1.25 (*m*, 16 H); 1.08 (*s*, Me₃C); 0.88 (*t*, *J* = 6.3, Me). ¹³C-NMR (CDCl₃, 75 MHz): 135.50 (*4d*); 133.27 (*d*, C(4)); 132.91, 132.86 (2*s*); 129.75 (2*d*); 127.69 (*4d*); 127.20 (*d*, C(5)); 93.71 (*t*, OCH₂O); 75.45 (*d*, C(3)); 70.56 (*t*, C(6)); 70.35 (*t*, C(1')); 66.59 (*d*, C(2)); 63.73 (*t*, C(1)); 55.66 (*q*, MeO); 32.03, 29.85, 29.75, 29.64, 29.46, 26.31, 22.83 (several *t*); 26.86 (*q*, Me₃C); 19.27 (*s*, Me₃C); 14.28 (*q*, Me). HR-ESI-MS: 632.3848 (100, [M + Na]⁺, C₃₃H₅₃N₃NaO₄Si⁺; calc. 632.3854).

(4E)-2-Azido-2,4,5-trideoxy-3-O-(methoxymethyl)-6-O-undecyl-D-erythro-hex-4-enitol (**7**). A soln. of **6** (773 mg, 1.27 mmol) in THF (20 ml) was treated with Bu₄NF·3 H₂O (801 mg, 2.54 mmol) and AcOH (0.14 ml, 2.54 mmol) at 0°, warmed to r.t., stirred for 4 h, diluted with H₂O, and extracted with CH₂Cl₂ (3 × 100 ml). The combined org. layers were dried (Na₂SO₄), and evaporated. FC (AcOEt/hexane 3 : 7) gave **7** (449 mg, 95%). Colourless oil. *R*_f (AcOEt/hexane 3 : 7) 0.19. [α]_D²⁵ = –104.7 (*c* = 2.0, CHCl₃). IR (CHCl₃): 3448w (br.), 3018m, 2929s, 2856s, 2130m, 2106s, 1599w, 1466w, 1372w, 1354w, 1298w, 1274w, 1219m, 1152m, 1099m, 1068w, 1033m, 974w, 915w. ¹H-NMR (CDCl₃, 300 MHz): 5.87 (*ddt*, *J* = 15.6, 5.1, 0.6, H–C(5)); 5.64 (*ddt*, *J* = 15.6, 8.1, 1.2, H–C(4)); 4.69 (*d*, *J* = 6.9, 1 H, OCH₂O); 4.55 (*d*, *J* = 6.6, 1 H, OCH₂O); 4.21 (*dd*, *J* = 7.8, 5.4, H–C(3)); 3.99 (*dd*, *J* = 5.1, 1.5, 2 H–C(6)); 3.76 (*dd*, *J* = 11.2, 4.2, H–C(1)); 3.71 (*dd*, *J* = 11.2, 6.0, H'–C(1)); 3.55 (*td*, *J* = 5.7, 4.2, H–C(2)); 3.41 (*t*, *J* = 6.6, 2 H–C(1')); 3.38 (*s*, MeO); 2.40 (br. *s*, HO–C(1)); 1.62–1.52 (*m*, 2 H–C(2')); 1.34–1.23 (*m*, 16 H); 0.87 (*t*, *J* = 6.9, Me). ¹³C-NMR (CDCl₃, 75 MHz): 133.48 (*d*, C(4)); 127.17 (*d*, C(5)); 93.80 (*t*, OCH₂O); 76.29 (*d*, C(3)); 70.75 (*t*, C(6)); 70.22 (*t*, C(1')); 66.16 (*d*, C(2)); 62.12 (*t*, C(1)); 55.79 (*q*, OMe); 31.96, 29.75, 29.67, 29.56, 29.40, 26.33, 22.76 (several *t*); 14.21 (*q*, Me). HR-MALDI-MS: 394.2669 (100, [M + Na]⁺, C₁₉H₃₇N₃NaO₄⁺; calc. 394.2676). Anal. calc. for C₁₉H₃₇N₃O₄ (371.52): C 61.43, H 10.04, N 11.31; found: C 61.60, H 10.05, N 11.35.

(4E)-2-Azido-2,4,5-trideoxy-3-O-(methoxymethyl)-1-O-[(trifluoromethyl)sulfonyl]-6-O-undecyl-D-erythro-hex-4-enitol (**3**). Tf₂O (60 μl, 0.37 mmol) was added dropwise to a soln. of **7** (113 mg, 0.31 mmol) and pyridine (60 μl, 0.92 mmol) in dry CH₂Cl₂ (8 ml), cooled to –78°. The mixture was warmed to –20° within 3.5 h, diluted with NH₄Cl soln., and extracted with CH₂Cl₂ (3 × 50 ml). The combined org. layers were dried (Na₂SO₄) and evaporated to give pale yellow oily **3**, which was used for the next step without further purification. *R*_f (AcOEt/hexane 2 : 8) 0.46. ¹H-NMR (CDCl₃, 300 MHz): 5.94 (*ddt*, *J* = 15.6, 5.1, 0.9, H–C(5)); 5.62 (*ddt*, *J* = 15.6, 8.1, 1.5, H–C(4)); 4.71 (*d*, *J* = 6.6, 1 H, OCH₂O); 4.63 (*dd*, *J* = 10.6, 3.5, H–C(1)); 4.53 (*d*, *J* = 6.9, 1 H, OCH₂O); 4.48 (*dd*, *J* = 10.5, 7.8, H'–C(1)); 4.22 (*dd*, *J* = 7.8, 5.7, H–C(3)); 4.02 (*dd*, *J* = 5.1, 1.8, 2 H–C(6)); 3.80 (*ddd*, *J* = 7.8, 6.0, 3.6, H–C(2)); 3.43 (*t*, *J* = 6.6, 2 H–C(1')); 3.38 (*s*, MeO); 1.61–1.54 (*m*, 2 H–C(2')); 1.38–1.20 (*m*, 16 H); 0.88 (*t*, *J* = 6.3, Me). ¹³C-NMR (CDCl₃, 75 MHz): 135.23 (*d*, C(4)); 125.43 (*d*, C(5)); 93.67 (*t*, OCH₂O); 75.24 (*d*, C(3)); 74.44 (*t*, C(1)); 70.95 (*t*, C(6)); 69.92 (*t*, C(1')); 63.17 (*d*, C(2)); 56.00 (*q*, MeO); 31.96, 29.75, 29.67, 29.56, 29.40, 26.33, 22.76 (several *t*); 14.21 (*q*, Me). ¹⁹F-NMR (CDCl₃): –74.22 (*s*, CF₃).

(4E)-1-S-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-2-azido-2,4,5-trideoxy-3-O-(methoxymethyl)-1-thio-6-O-undecyl-D-erythro-hex-4-enitol (**8**). A soln. of **2** (95 mg, 0.25 mmol) and crude **3** in dry CH₂Cl₂ (10 ml) was treated with Hünig's base (63 μl, 0.38 mmol), stirred at r.t. for 2.5 h, diluted with NH₄Cl, and extracted with CH₂Cl₂. The combined org. layers were dried (Na₂SO₄) and evaporated. FC (AcOEt/hexane 1 : 1) gave **8** (136 mg, 75%). Colourless oil. *R*_f (AcOEt/hexane 7 : 3) 0.20. [α]_D²⁵ = +38.5 (*c* = 1.75, CHCl₃). IR (CHCl₃): 3018w, 2955w, 2929m, 2855m, 2120m, 1747s, 1680m, 1601w, 1511m, 1466w, 1371m, 1262s, 1250s, 1169w, 1151w, 1085s, 1053m, 1029m, 978w, 914w, 862w, 821w. ¹H-NMR (CDCl₃, 300 MHz): 5.85 (*ddt*, *J* = 15.6, 5.1, 0.9, H–C(5)); 5.73 (*d*, *J* = 8.7, NH); 5.59 (*d*, *J* = 5.4, H–C(1''')); 5.58 (*ddt*, *J* = 15.6, 8.1, 1.5, H–C(4)); 5.38 (*dd*, *J* = 3.3, 1.2, H–C(4''')); 5.03 (*dd*, *J* = 12.0, 3.3, H–C(3''')); 4.76 (*ddd*, *J* = 12.0, 8.7, 5.4, H–C(2''')); 4.68 (*d*, *J* = 6.9, 1 H, OCH₂O); 4.56 (*td*, *J* = 6.6, 0.9, H–C(5''')); 4.53 (*d*, *J* = 6.3, 1 H, OCH₂O); 4.13 (*dd*, *J* = 11.4, 6.0, H–C(6''')); 4.15 (*dd*, *J* = 8.1, 4.5, H–C(3)); 4.04 (*dd*, *J* = 11.4, 6.9, H'–C(6''')); 3.98 (*dd*, *J* = 5.4, 1.2, 2 H–C(6)); 3.68 (*dt*, *J* = 9.6, 4.2, H–C(2)); 3.39 (*t*, *J* = 6.6, 2 H–C(1')); 3.36 (*s*, MeO); 2.79 (*dd*, *J* = 13.8, 4.2, H–C(1)); 2.67 (*dd*, *J* = 13.8, 9.6, H'–C(1)); 2.14 (*s*, AcNH); 2.04, 1.99, 1.97 (3*s*, 3 AcO); 1.60–1.51 (*m*, 2 H–C(2')); 1.35–1.24 (*m*, 16 H); 0.86 (*t*, *J* = 6.3, Me). ¹³C-NMR (CDCl₃, 75 MHz): 170.82 (*s*, NHAc); 170.21, 170.04, (2*s*, 3 AcO); 134.38 (*d*, C(4)); 126.19 (*d*, C(5)); 93.66 (*t*, OCH₂O); 86.46 (*d*, C(1''')); 77.83 (*d*, C(3)); 70.85, 70.20 (2*t*,

C(6), C(1''); 68.29, 67.59, 67.28 (3d, C(3''), C(4''), C(5'')); 65.81 (d, C(2)); 61.93 (t, C(6'')); 55.86 (q, OMe); 48.43 (d, C(2'')); 32.82, 31.99, 29.78, 29.70, 29.59, 29.43, 26.26, 22.78 (several t); 23.39 (q, AcNH); 20.84, 20.80, 20.77 (3q, 3 AcO); 14.24 (q, Me). HR-ESI-MS: 739.3548 (100, $[M + Na]^+$, $C_{33}H_{56}N_4NaO_{11}S^+$; calc. 739.3559). Anal. calc. for $C_{33}H_{56}N_4O_{11}S$ (716.89): C 55.29, H 7.87, N 7.82; S 4.47; found: C 55.14, H 8.07, N 7.70; S 4.44.

(4E)-1-S-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-galactopyranosyl)-2,4,5-trideoxy-3-O-(methoxymethyl)-2-(octadecanoylamino)-1-thio-6-O-undecyl-D-erythro-hex-4-enitol (**9**). A suspension of **8** (100 mg, 0.14 mmol) and polymer-bound PPh_3 (352 mg, 0.56 mmol) in dioxane (5 ml) was heated at 80° for 1 h. The temp. was then reduced to 60°, stearic anhydride (154 mg, 0.28 mmol) was added, the mixture was stirred for 48 h, filtered, and the filtrate was evaporated. FC (AcOEt/hexane 6:4) gave **9** (115 mg, 86%). White crystals. R_f (AcOEt/hexane 7:3) 0.31. M.p. 66–68.5°. $[\alpha]_D^{25} = +30.8$ ($c = 1.0$, $CHCl_3$). IR ($CHCl_3$): 3426w, 3019m, 2927s, 2855s, 1747s, 1676m, 1602w, 1511m, 1466w, 1371m, 1239m, 1151w, 1084m, 1050m, 1031m, 977w, 914w. 1H -NMR ($CDCl_3$, 300 MHz): 6.01 (d, $J = 9.0$, AcNH); 5.83 (dd, $J = 15.6, 5.1, 0.9$, H-C(5)); 5.60 (d, $J = 8.7$, HN-C(2)); 5.58 (ddt, $J = 15.6, 6.9, 1.8$, H-C(4)); 5.45 (d, $J = 5.1$, H-C(1'')); 5.38 (dd, $J = 3.0, 1.2$, H-C(4'')); 5.01 (dd, $J = 12.0, 3.3$, H-C(3'')); 4.77 (ddd, $J = 12.0, 9.0, 5.4$, H-C(2'')); 4.65 (d, $J = 6.6, 1$ H, OCH_2O); 4.56 (td, $J \approx 6.6, 0.9$, H-C(5'')); 4.54 (d, $J = 6.9, 1$ H, OCH_2O); 4.29–4.21 (m, H-C(2)); 4.17 (dd, $J = 11.4, 6.0$, H-C(6'')); 4.14 (t, $J \approx 6.2$, H-C(3)); 4.07 (dd, $J = 11.4, 6.6$, H'-C(6'')); 3.95 (br. d, $J = 5.1, 2$ H-C(6)); 3.38 (t, $J = 6.9, 2$ H-C(1')); 3.37 (s, MeO); 3.00 (dd, $J = 14.1, 7.5$, H-C(1)); 2.83 (dd, $J = 13.8, 3.9$, H'-C(1)); 2.20–2.08 (AB, 2 H-C(2'')); 2.16 (s, AcNH); 2.04, 2.00, 1.97 (3s, 3 AcO); 1.62–1.51 (m, 2 H-C(2'), 2 H-C(3'')); 1.38–1.19 (m, 44 H); 0.87 (t, $J = 6.6, 2$ Me). ^{13}C -NMR ($CDCl_3$, 75 MHz): 172.66 (s, C(1'')); 170.82, 170.19, 170.05 (3s, 3 AcO); 169.92 (s, AcNH); 132.65 (d, C(4)); 128.01 (d, C(5)); 94.56 (t, OCH_2O); 86.79 (d, C(1'')); 78.01 (d, C(3)); 70.83, 70.35 (2t, C(6) C(1')); 68.35, 67.69, 67.25 (3d, C(3''), C(4''), C(5'')); 61.79 (t, C(6'')); 56.02 (q, MeO); 52.21 (d, C(2)); 48.41 (d, C(2'')); 36.89, 33.08, 32.02, 29.82, 29.75, 29.67, 29.51, 29.47, 26.29, 25.75, 22.81 (several t); 23.47 (q, AcNH); 20.86 (q, 3 AcO); 14.24 (q, 2 Me). HR-MALDI-MS: 979.6244 (84, $[M + Na]^+$, $C_{51}H_{92}N_2NaO_{12}S^+$; calc. 979.6263). Anal. calc. for $C_{51}H_{92}N_2O_{12}S$ (957.36): C 63.98, H 9.69, N 2.93; found: C 64.19, H 9.68, N 2.96.

(4E)-1-S-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-galactopyranosyl)-2,4,5-trideoxy-2-(octadecanoylamino)-1-thio-6-O-undecyl-D-erythro-hex-4-enitol (**10**). An ice-cold soln. of **9** (90 mg, 0.09 mmol) in dry MeCN was treated with Tf_2O (32 μ l, 0.19 mmol), slowly warmed to r.t. in 2 h, diluted with sat. NH_4Cl soln., and extracted with CH_2Cl_2 (3 $\times$ 50 ml). The combined org. layers were dried (Na_2SO_4) and evaporated. FC (AcOEt/hexane 9:1) gave **10** (75 mg, 87%). White crystals. R_f (AcOEt/hexane 9:1) 0.23. M.p. 95–98°. $[\alpha]_D^{25} = +55.9$ ($c = 2.25$, $CHCl_3$). IR ($CHCl_3$): 3573w, 3485w, 3419w, 3021w, 2928m, 2855w, 1747m, 1675w, 1604w, 1512w, 1466w, 1372w, 1266s, 1170m, 1085w, 1044m, 1037m, 978 w, 929w. 1H -NMR ($CDCl_3$, 300 MHz): 6.14 (d, $J = 8.7$, AcNH); 5.87 (br. dt, $J = 15.6, 5.3$, H-C(5)); 5.75 (d, $J = 8.7$, HN-C(2)); 5.71 (br. dd, $J = 15.6, 5.7$, H-C(4)); 5.50 (d, $J = 5.4$, H-C(1'')); 5.37 (dd, $J = 3.0, 0.9$, H-C(4'')); 5.00 (dd, $J = 11.7, 3.3$, H-C(3'')); 4.74 (ddd, $J = 11.8, 8.6, 5.1$, H-C(2'')); 4.53 (br. t, $J = 6.6$, H-C(5'')); 4.29 (br. t, $J \approx 4.9$, H-C(3)); 4.15 (dd, $J = 11.6, 6.2$, H-C(6'')); 4.15–4.05 (m, H-C(2)); 4.08 (dd, $J = 11.3, 6.8$, H'-C(6'')); 3.95 (br. d, $J = 5.4, 2$ H-C(6)); 3.39 (t, $J = 6.9, 2$ H-C(1')); 3.00 (dd, $J = 14.0, 8.0$, H-C(1)); 2.80 (dd, $J = 14.0, 4.1$, H'-C(1)); 2.22–2.12 (AB, 2 H-C(2'')); 2.15 (s, AcNH); 2.05, 1.99, 1.97 (3s, AcO); 1.64–1.50 (m, 2 H-C(2'), 2 H-C(3'')); 1.36–1.18 (m, 44 H); 0.87 (t, $J = 6.8, 2$ Me). ^{13}C -NMR ($CDCl_3$, 75 MHz): 173.86 (s, C(1'')); 170.80 (s, AcNH); 170.33, 170.07, 169.99 (3s, 3 AcO); 132.54 (d, C(4)); 130.05 (d, C(5)); 86.75 (d, C(1'')); 73.51 (d, C(3)); 70.87, 70.45 (2t, C(6) C(1')); 68.28, 67.63, 67.15 (3d, C(3''), C(4''), C(5'')); 61.89 (t, C(6'')); 54.30 (d, C(2)); 48.47 (d, C(2'')); 36.73 (t); 32.65 (t); 31.98 (2t); 29.78–29.42 (several t); 26.25 (t); 25.78 (t); 23.39 (q, AcNH); 22.77 (2t); 20.90, 20.82, 20.77 (3q, 3 AcO); 14.21 (q, 2 Me). HR-MALDI-MS: 935.5984 (100, $[M + Na]^+$, $C_{49}H_{88}N_2NaO_{11}S^+$; calc. 935.6001). Anal. calc. for $C_{49}H_{88}N_2O_{11}S$ (913.29): C 64.44, H 9.71, N 3.07, S 3.51; found: C 64.18, H 9.74, N 3.11, S 3.36.

(4E)-1-S-(2-Acetamido-2-deoxy- α -D-galactopyranosyl)-2,4,5-trideoxy-2-(octadecanoylamino)-1-thio-6-O-undecyl-D-erythro-hex-4-enitol (**1**). A soln. of **10** (70 mg, 0.076 mmol) in 6 ml of 2M NH_3 in MeOH was heated to 40° for 4.5 h, and then evaporated. FC (MeOH/ CH_2Cl_2 3:17) gave **1** (58 mg, 96%). R_f (CH_2Cl_2 /MeOH 9:1) 0.26. M.p. 207–209°. $[\alpha]_D^{25} = +56.3$ ($c = 1.08$, pyridine). IR ($CHCl_3$): 3407m (br.), 3289s, 3092w, 2953m, 2919s, 2850s, 1647s, 1551s, 1468m, 1435m, 1376m, 1298w, 1213w, 1168w,

1116m, 1084m, 1049m, 1020w, 977w, 960w. ¹H-NMR (CD₃OD, 300 MHz): 7.98 (d, *J* = 7.5, 0.4 H, AcNH); 7.88 (d, *J* = 9.0, 0.6 H, HN–C(2)); 5.78 (br. dt, *J* = 15.3, 5.2, H–C(5)); 5.71 (br. dd, *J* = 15.8, 5.9, H–C(4)); 5.52 (d, *J* = 5.4, H–C(1''')); 4.40 (dd, *J* = 11.4, 5.4, H–C(2''')); 4.21 (ddd, *J* = 6.0, 4.8, 0.9, H–C(3)); 4.05 (br. t, *J* = 6.5, H–C(5'')); 4.03–3.97 (m, H–C(2)); 3.94 (d, *J* = 4.5, 2 H–C(6)); 3.86 (d, *J* = 2.4, 2 H–C(4'')); 3.79 (dd, *J* = 11.7, 7.2, H–C(6'')); 3.73 (dd, *J* = 11.7, 5.1, H'–C(6'')); 3.65 (dd, *J* = 11.6, 3.2, H–C(3'')); 3.41 (t, *J* = 6.6, 2 H–C(1')); 2.91 (dd, *J* = 13.8, 3.3, H–C(1)); 2.82 (dd, *J* = 13.7, 8.6, H'–C(1)); 2.18 (t, *J* = 7.4, 2 H–C(2'')); 1.96 (s, AcNH); 1.64–1.50 (m, 2 H–C(2'), 2 H–C(3'')); 1.36–1.26 (m, 44 H); 0.89 (t, *J* = 6.8, 2 Me). ¹³C-NMR ((D₅)pyridine, 75 MHz): 173.46 (s, C(1'')); 170.44 (s, AcNH); 134.08 (d, C(4)); 128.69 (d, C(5)); 87.77 (d, C(1'')); 73.61 (d, C(5'')); 73.35 (d, C(3)); 71.08, 70.29 (2t, C(6), C(1')); 70.17, 69.41 (2d, C(3''), C(4'')); 62.75 (t, C(6'')); 55.26 (d, C(2)); 52.24 (d, C(2'')); 36.76 (t); 33.46 (t); 32.07 (2t); 30.21–29.56 (several t); 26.57 (t); 25.34 (t); 23.14 (q, AcNH); 22.89 (2t); 14.26 (2q, Me). HR-MALDI-MS: 809.5669 (100, [*M* + Na]⁺, C₄₃H₈₂N₂NaO₈S⁺; calc. 809.5684). Anal. calc. for C₄₃H₈₂N₂O₈S · H₂O (805.2001): C 64.14, H 10.52, N 3.48; found: C 64.10, H 10.69, N 3.45.

2. *Biological Tests. Phosphorylation of Ceramide Analogue by CerK.* Activity of CerK on C8-ceramide (Cayman) and the ceramide analogue **1** was measured according to published procedures [55] using 180 μM of either C8 ceramide or analogue **1** in the direct assay (Fig. 1, a), and 20 μM C8-ceramide and 100 μM **1** in the competitive assay (Fig. 1, b).

T-Cell Clones and APC. Human CD1d-restricted iNKT cell clones were derived and cultivated as described in [60]. Human CD1d-transfected C1R or THP1 cells were used as APC in the experiments.

Antigen-Presentation Assays. Human CD1d transfectants (2.5 × 10⁴/well) in RPMI-1640 medium containing 10% FCS were incubated at 37° with sonicated compounds at the concentrations indicated in the Figures in the presence of T cells (7.5 × 10⁴/well). Supernatants were harvested after 24 h, and released cytokines were measured by ELISA.

Plate-Bound Human CD1d Competition Assay. Plates were coated overnight at r.t. with 10 μg/ml BIR1.4 monoclonal antibody (mAb). Soluble recombinant human CD1d purified by isoelectric focusing was incubated overnight at r.t. on BIR1.4-coated plates at twofold molar concentration of BIR1.4 mAb. Sonicated compounds were added 4.5 h before αGalCer to plate-bound human CD1d and left overnight at r.t. to allow competition. Then, T cells (1.5 × 10⁵/well) were plated in RPMI-1640 medium containing 5% human serum and 100 U/ml human IL-2 after extensive washing. Supernatants were harvested after 24 h of incubation at 37°, and released cytokines were measured by ELISA.

ELISA. Plates coated with 8D4-8 (anti-human IL-4 mAb, BD), MAb1 (anti-human TNF-α mAb, BD), 6804 (anti-human GM-CSF mAb, R&D), or HB-8700 (anti-human IFN-γ mAb, ATCC) were blocked and then incubated with the supernatants of the antigen presentation assays.

Detection of human IL-4, human TNF-α, or human IFN-γ was performed using MP4-25D2 (anti-human IL-4 biotin-labelled mAb, BD), MAb11 (anti-human TNF-α biotin-labelled mAb, BD), 3209 (anti-human GM-CSF biotin-labelled mAb, R&D), or γ69 [61] (anti-human IFN-γ biotin-labelled mAb, provided by G. Garotta) with streptavidin-HRP (Zymed, Invitrogen), and *o*-phenylenediamine dihydrochloride (Sigma, according to the manufacturer's instructions) as substrate. OD_{490 nm} was read on a spectrophotometer (Molecular Devices, Sunnyvale, CA) and converted to concentrations using the SoftMax Pro 5 program by comparison to standards of human IL-4 (human IL-4 secreting X63, kind gift of U. Grawunder), recombinant human TNF-α (Immunokontakt), and recombinant human IFN-γ (BenderMedSystems).

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