Total Enantioselective Synthesis and In Vivo Biological Evaluation of a Novel Fluorescent BODIPY α-Galactosylceramide

Yen Vo-Hoang,[a] Laurent Micouin,*[a] Catherine Ronet,[b] Gabriel Gachelin,[b] and Martine Bonin*[a]

Natural killer T (NKT) cells are a distinct subset of mature lymphocytes endowed with features of activated and regulatory T cells. α-Galactosylceramides (α-GalCers), the synthetic prototype of which is KRN7000, are the only natural reagents recognised by the T-cell receptor of NKT cells. The α-GalCer-activated NKT cells promptly release IFNγ and IL-4 (IFN = interferon; IL = interleukin) and undergo apoptotic death within hours. In mice, activated NKT cells are responsible for antitumour activity and protection against autoimmune diseases. KRN7000 can thus be considered as the root of a family of novel immunoregulatory drugs. To get insights into the in vivo behaviour of α-galactosylceramides, an original fluorescent derivative has been prepared by following a convergent synthetic scheme. This strategy allows the introduction of different acyl chains, carbohydrate residues and various labels in the final steps of the synthesis. The fluorescent BODIPY probe derived from a versatile glycolipid precursor is as active as KRN7000 for inducing apoptosis of liver NKT cells. Fluorescence was detected in peritoneal macrophages and splenic antigen-presenting cells, in Kupffer-like cells in the liver, but not in lymphocytes.

KEYWORDS:

asymmetric synthesis · fluorescent probes · glycolipids · immunoregulation · natural killer T cells

Introduction

Natural and synthetic α-galactosylceramides (α-GalCers) are receiving increasing attention.[1] These molecules are the only reagents known to specifically activate "natural killer T cells" (NKT cells), a subpopulation of regulatory T cells that plays a pivotal role in immune system homeostasis.[2] The distinctive role of α-GalCer-activated NKT cells in a variety of autoimmune pathologies, which include multiple sclerosis,[3] autoimmune diabetes[4] and experimental encephalomyelitis,[5] and in tumour rejection,[6] organ transplants[7] and atherosclerosis,[8] has recently been evidenced.[7] Molecules belonging to the α-galactosylceramide family may thus evolve into useful immunomodulatory drugs.

The first α-galactosylceramides were isolated from marine sponges[9] and were later synthesised by Koezuka et al., who developed potent glycolipid analogues such as KRN7000 (**1**; Scheme 1).[10] Other synthetic α-galactosylceramide derivatives such as **3** and **4** (Scheme 1) are now available and these significantly activate NKT cells.[11] Immobilised, biotinylated derivatives such as AGL-592 (**3**) have been used to probe the specificity of the α derivatives versus that of the β derivatives in the activation of NKT cells in vitro.[12]

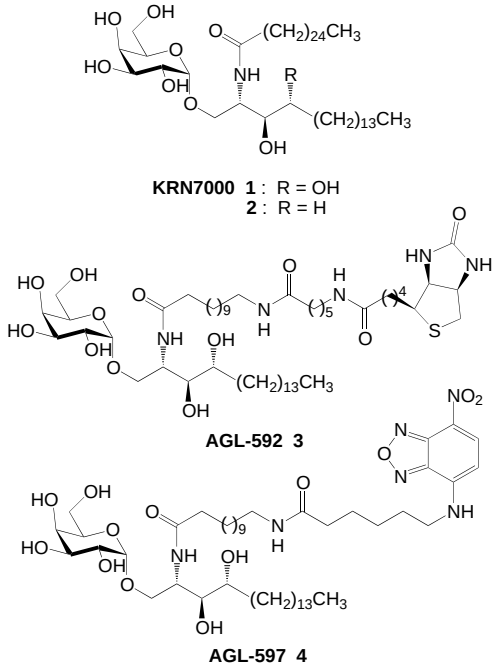
In a model of murine intestinal inflammation in which α-GalCer-activated NKT cells play a beneficial role, the NBD analogue **4** was detected on the colonic surface epithelium.[13] However, despite the potential interest of the α-GalCer derivatives, their in vivo behaviour, cell storage, metabolism and clearance are far from fully described. Therefore, the synthesis of other pharmacologically active and labelled analogues is essential for further in vivo studies. Also, more versatile synthetic pathways must be designed to easily create families of related molecules and probes.[1, 11]

We have developed a versatile synthetic route which was used to construct the first biologically active KRN7000 analogue **2**.[1a] Once the biological activity of this molecule had been ascertained, the same route was used to construct a related fluorescent glycolipid **5** (see below).

The introduction of a fluorescent dye into a bioactive molecule may alter some of the biological properties of the latter. Nevertheless, glycolipids labelled with the fluorophore boron dipyrromethene difluoride (BODIPY) are widely used as potent tools for studying metabolism and glycolipid traffic in

[a] *Dr. L. Micouin, Dr. M. Bonin, Y. Vo-Hoang*
Laboratoire de Chimie Thérapeutique
UMR 8638 associée au CNRS
et à l'Université René Descartes
Faculté des Sciences Pharmaceutiques et Biologiques
4 avenue de l'observatoire
75270 Paris cedex 06 (France)
Fax: (+33) 1432-91403
E-mail: bonin@pharmacie.univ-paris5.fr

[b] *C. Ronet, Dr. G. Gachelin*
Unité de Biologie Moléculaire du Gène
INSERM U277, Département d'Immunologie
Institut Pasteur, 25 rue du Docteur Roux
75015, Paris (France)
E-mail: ggachel@pasteur.fr



Scheme 1. *Structures of biologically active α-GalCer analogues.*

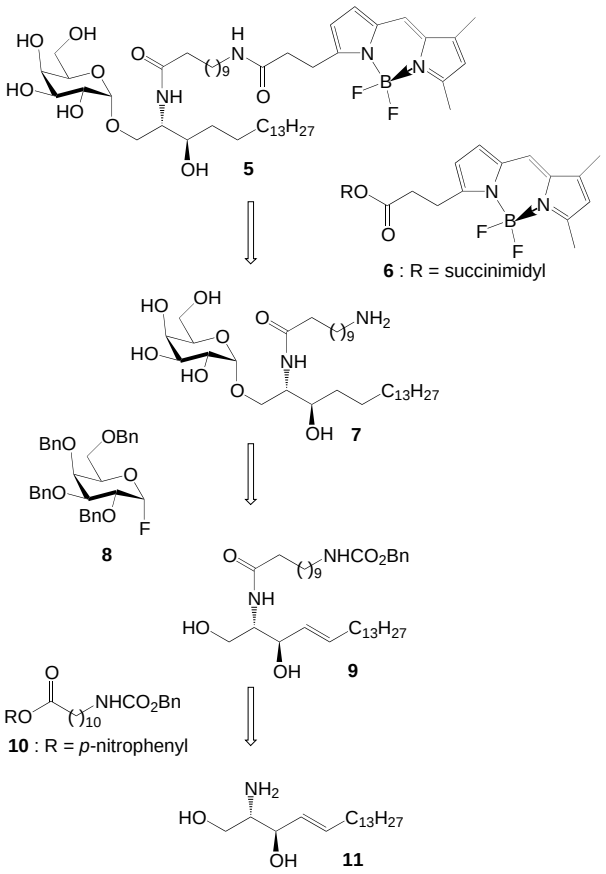
animal cells.[14] The strong electronic transitions of the label (at about 500 nm) are also safely shifted from potential overlapping bands caused by proteins and lipid membranes.

On the basis of the wide use of BODIPY and of the known structure – activity relationships for α-galactosylceramides,[10, 15] we prepared the novel α-GalCer-BODIPY probe (**5**).[16] To the best of our knowledge this fluorescent group has never before been introduced into α-galactosylceramide derivatives. In contrast with previous strategies in which the label groups were introduced into natural degradation material through a tether,[12a, 13a] we decided to apply our convergent synthetic route to the preparation of the versatile intermediate **7** (Scheme 2). Consideration of active analogues **3** and **4**, which have a fluorescent group at the end of the fatty acyl chain, led us to introduce the BODIPY group in the last step of the synthesis through the terminal amine function.

In such an approach, the relative and absolute configurations of the central aminodiol motif remain unchanged, and the glycosidic moiety and the terminal labelling groups can be changed in the last steps of the synthesis. Herein, we report the practical preparation of the novel α-GalCer fluorescent probe **5**, the in vivo investigation of its biological activity and the identification of the cells able to capture it.

Results and Discussion

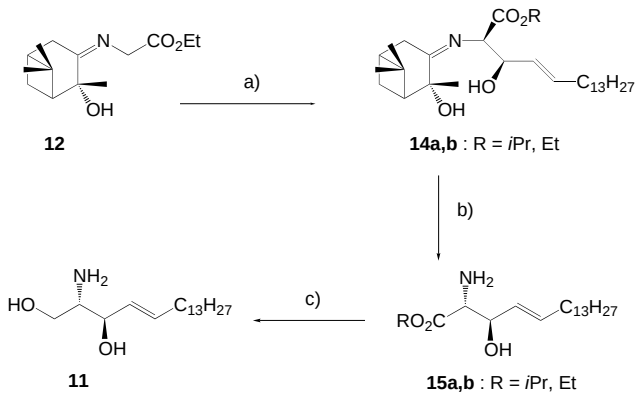
The synthesis of the designed glycolipid precursor **7** follows a convergent process in which the diastereoselective preparation of D-*erythro*-sphingosine (**11**) is followed by the successive couplings with the fatty aminoacyl chain **10** and the galactosyl donor **8** (Scheme 2).



Scheme 2. *Retrosynthetic analysis of the fluorescent α-GalCer – BODIPY probe **5**.*

Optimised conditions for the diastereoselective synthesis of D-*erythro*-sphingosine (11)

The iminoglycinate **12** and (*E*)-2-hexadecenal (**13**) were prepared according to literature procedures with some minor variations to improve process efficiency (Scheme 3).[17] The use of the $ClTi(OiPr)_3/NEt_3$ system instead of $ClTi(OEt)_3$ for the generation of the reactive titanium enolate allowed easy conversion of iminoester **12** and proved more convenient for multigram-scale

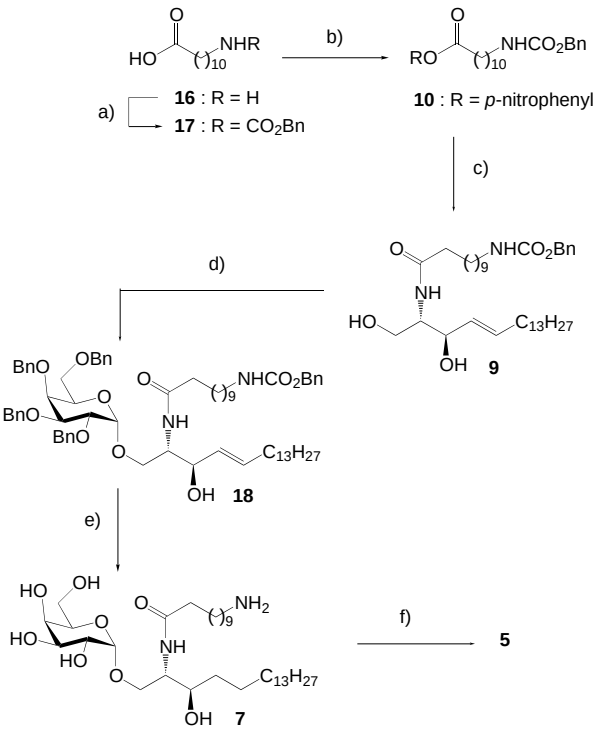


Scheme 3. *Preparation of D-erythro-sphingosine (**11**). a) $ClTi(OiPr)_3$, (E)-2-hexadecenal (**13**), NEt_3, CH_2Cl_2, 0 °C, 91% yield of **14a,b**; b) 1 M HCl, THF, 20 °C, 77% yield of **15a,b**; c) $LiBH_4$, THF, MeOH, 20 °C, 84%.*

syntheses. The NMR spectra of the products indicated that only one diastereomer was formed (>97%) as a mixture of ethyl and isopropyl esters **14a**,**b** in a 7:3 ratio. After classical acid hydrolysis, direct hydride reduction of the ester mixture **14a**,**b** was performed with a 2M suspension of $LiBH_4$ in a mixture of tetrahydrofuran (THF) and methanol at 20°C. The known D-*erythro*-sphingosine (**11**) was thus obtained in a total yield of 59% starting from the chiral nonracemic iminoester **12**.

Preparation of the new protected spacer arm 10 and the corresponding ceramide 9

Compound **10** was prepared from commercially available 11-aminoundecanoic acid (**16**) in a two-step sequence involving *N*-carbamate formation and *para*-nitrophenylester derivatisation (Scheme 4). The benzyl derivative was chosen to allow a one-pot deprotection and final reduction step (Scheme 2). The coupling of sphingosine (**11**) with the activated ester **10** in the presence of a catalytic amount of 4-(dimethylamino)pyridine (DMAP) furnished ceramide **9** in 76% yield.



Scheme 4. *Preparation of the original α-GalCer – BODIPY,* ***5****. a)* $ClCO_2Bn$, CH_2Cl_2, *reflux, 15%; b)* p-*nitrophenol,* N,N′-*dicyclohexylcarbodiimide, DMAP,* CH_2Cl_2, *20°C, 47%; c)* ***11****, DMAP, THF, 20°C, 76%; d)* ***8****,* $SnCl_2$, $AgClO_4$, *molecular sieves,* Et_2O, *THF, 20°C, 37%; e)* H_2, *Pd/C, MeOH, 20°C, 85%; f)* ***6****, DMF, 20°C, 25%. Bn = Benzyl.*

Synthesis of amino-α-galactoceramide 7

The synthesis of the new glycolipid precursor **7** is shown in Scheme 4. The activated galactosyl donor **8** was prepared according to previous procedures in a five-step sequence starting from D-galactose.[18] Coupling of **8** with the ceramide **9** under Mukayama's conditions ($AgClO_4/SnCl_2$) in THF/Et_2O (9:1.5) provided the desired compound **18** albeit in only 37% isolated yield. Despite intensive investigations, this low yield could not be improved, mainly because of solubility problems.

Careful NMR investigations proved the total stereocontrol of the new glycosidic bond formation ($J(1,2) = 3$ Hz). Final deprotection of all benzyl groups and double bond reduction were achieved in one step under classical hydrogenolysis conditions to afford the desired glycolipid **7** in pure form (85% yield). The synthetic sequence described above provided the new glycolipid analogue **7** in three steps and an overall yield of 24% starting from sphingosine (**11**).

Synthesis of the BODIPY fluorescent probe 5

The BODIPY fluorophore was coupled with compound **7** through its succinimidyl activated ester **6**. Final HPLC purification of the crude material afforded the desired fluorescent probe **5** in 25% yield.

Biological activity of the fluorescent probe 5

The biological activity of molecule **5** was probed in vivo and compared to that of KRN7000 (**1**) by administering the reagents by intraperitoneal (IP) injection into 8-week-old C57BL/6 mice and monitoring the activation-induced disappearance of liver NKT cells.[19] This test was used because it reflects the overall in vivo activation/apoptosis of NKT cells, with no prejudice for the type of cytokine released, which may itself depend upon the nature of the acyl chains.[20b]

Thus, 1, 5 or 10 μg of fluorescent derivative **5** was administered by IP injection. A similar concentration of KRN7000 (**1**) was injected as a control. Liver monocytes were isolated as described and analysed for the presence of $NK1.1^+$ TCR αβ int^+ cells (TCR = T-cell receptor). As little as 1 μg of molecule **5** caused a decrease of liver NKT cells from 33% to 0.5% (cf. KRN7000; Figure 1). Within this concentration range, the substituted molecule was therefore as active as KRN7000 (**1**) for inducing in vivo apoptosis of liver NKT cells. Similar results were obtained following intravenous (IV) injection (data not shown). Lower concentrations were not investigated.

The stability of the probe was tested by exposing **5** (10 μg) overnight at 37°C to undiluted mouse serum containing a suspension of total mouse liver leucocytes (shown to capture probe **5**, see below). The glycolipid material was extracted with chloroform/methanol and analysed by HPLC followed by fluorescence analysis of the fractions; no free BODIPY or degradation or substituted products were detected.

α-Galactosylceramides are usually administered by IP or IV injection. The cells that capture the molecules may vary depending on the method of administration. We therefore searched for fluorescent cells after IP or IV injection. We used animals injected with phosphate buffered saline (PBS) and unsubstituted BODIPY (BODIPY reagent **6**)-injected animals as controls. No detectable labelling of peritoneal, spleen and liver cells was observed following IP or IV injection of the latter. One

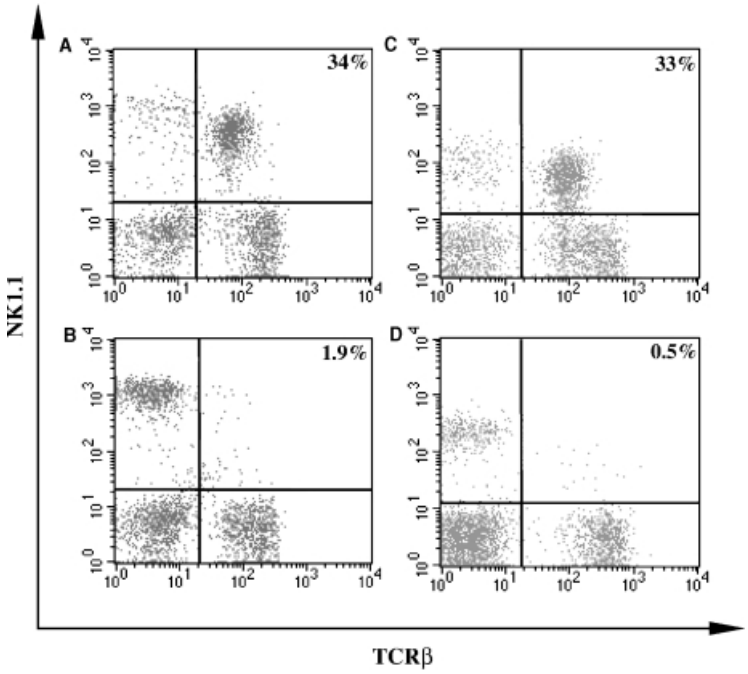


Figure 1. *Depletion of liver NKT cells by KRN7000 (**1**) and BODIPY probe **5**. Left panel: activity of KRN7000 (**1**): control (A) and injected mice (B). Right panel: activity of BODIPY probe **5**: control (C) and injected mice (D).*

hour after IP injection of probe **5**, cells were recovered by washing the peritoneal cavity. Nearly all macrophages were found to be stained. The staining was mostly associated with multiple intracytoplasmic vesicles or droplets (Figure 2).

Monocytes were recovered from the liver and the spleen of the animals treated by IP injection; adherent and nonadherent cells were collected separately. Fluorescence-activated cell sorter (FACS) analysis detected no positive cells in the spleen and liver. Thus, the IP-injected fluorescent glycolipid **5** was predominantly trapped by peritoneal macrophages.

The cells able to capture the BODIPY probe **5** following IV injection were also determined. Liver and spleen monocytes were isolated one hour after the injection. Adherent cells were sorted from nonadherent cells. The fraction of positive cells in each population was determined by image cytometry. Non-adherent cells (NK, NKT and lymphocytes) did not incorporate a significant amount of the labelled molecule **5**. About 10% of the adherent cells, predominantly Kupffer cells in the liver, macrophages and dendritic cells in the spleen, were found positive (Figure 3). The label was located in vesicles or droplets within the cells.

Interestingly, the staining of some spleen-adherent cells consisted of discrete patches on the cell membrane. This suggests that binding of the molecule to the membrane occurs before internalisation, rather than accumulation being the consequence of nonspecific endocytosis of glycolipid micelles.

The localisation of the fluorescent cells within the liver was examined in frozen sections counter-stained with Evan's blue (Figure 4). No staining of hepatocytes as such could be seen. The endothelial cells lining the blood vessels were not labelled either. The label was found in cells located within sinusoids. These cells looked like resident macrophages or Kupffer cells. The fluorescent dye was stored in vesicles in much the same way as in peritoneal macrophages, with some of the particles located next to the nucleus.

In summary, the fluorescent probe **5** was biologically active in the same typical test as KRN7000. The in vivo behaviour of the probe depends on the way it is injected: most of the IP-injected material is first captured by peritoneal macrophages; after IV injection, a significant concentration of fluorescent material was found in adherent liver and spleen cells. No overall staining was observed, which indicates that the label was taken up by minor subsets of cells. If the behaviour of probe **5** is representative of the family of α-galactosylceramides to which it belongs, we conclude that in vivo the α-GalCers are predominantly trapped by specialised cells rather than by random cell types.

The preferential capture of IP-injected material by peritoneal macrophages may appear difficult to reconcile with the effect on liver NKT cells. This can be accounted for in two ways: either an undetectable fraction of the material migrates towards the periphery, or loaded macrophages

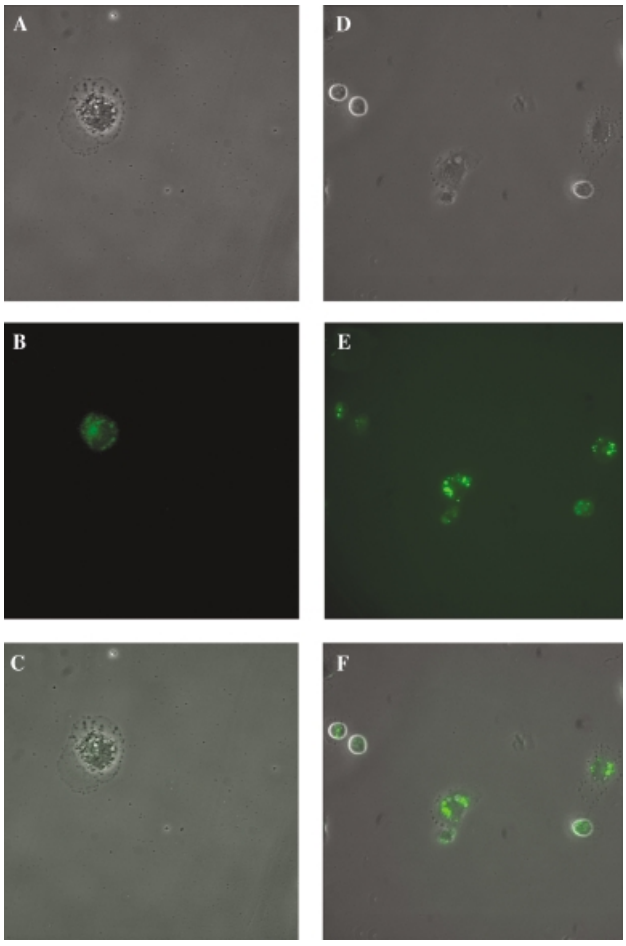


Figure 2. *Cellular and subcellular distribution of probe **5** in peritoneal macrophages of IP-injected animals. Left panel: PBS-injected mice: visible light (A), fluorescence (B), superimposition (C). Right panel: mice injected with BODIPY probe **5**: visible light (D), fluorescence (E), superimposition (F).*

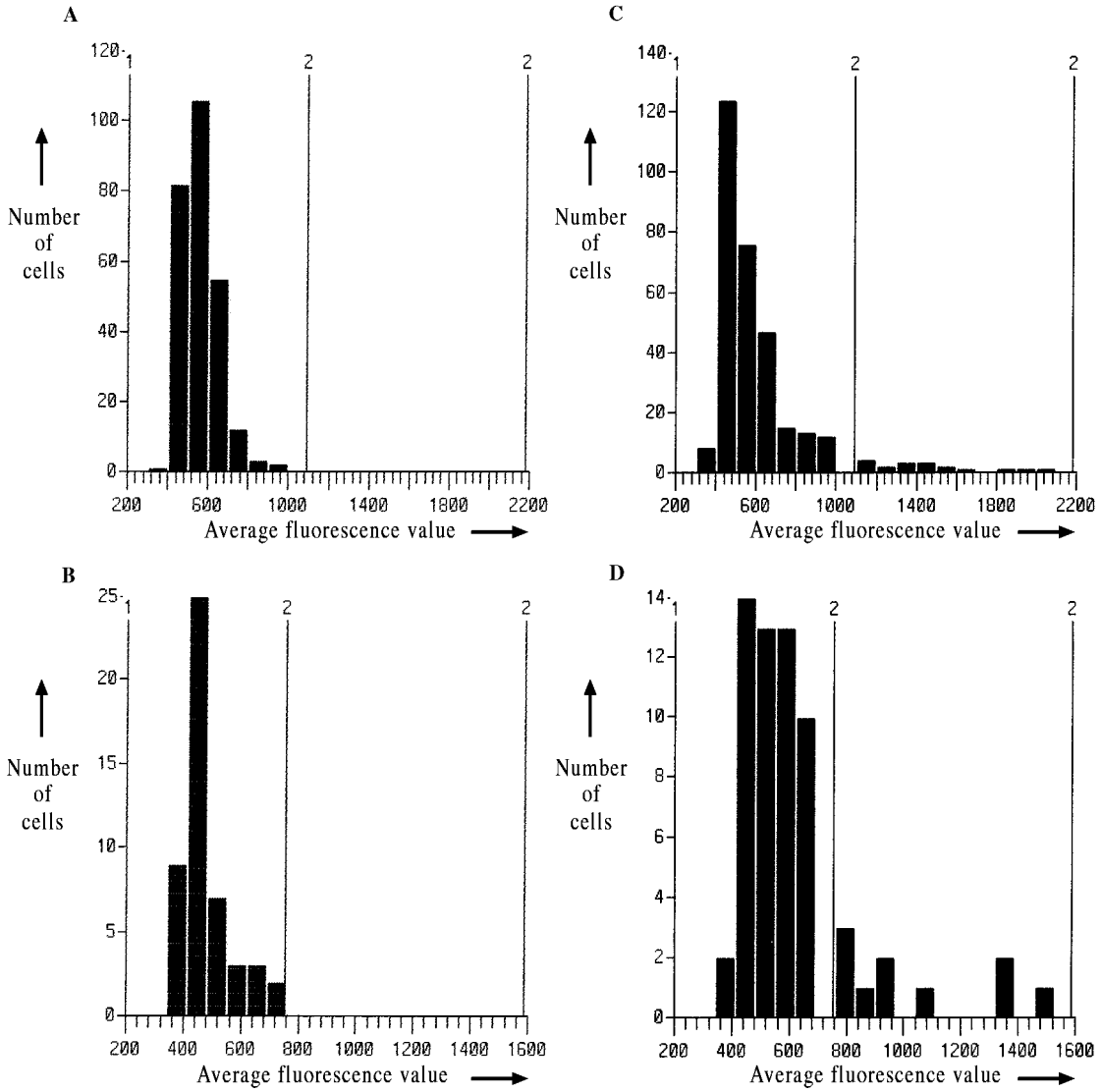


Figure 3. *Image cytometry of fluorescent adherent liver and spleen cells of animals IV injected with probe* ***5****. Left panel: PBS-injected mice: adherent liver cells (A), adherent spleen cells (B). Right panel: mice injected with BODIPY probe* ***5****: adherent liver cells (C), adherent spleen cells (D).*

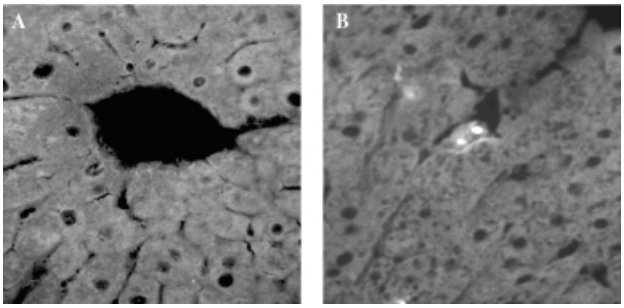


Figure 4. *Histological location of fluorescent cells on liver sections. The frozen sections (5 μm) of liver of mice IV injected with PBS (A) and BODIPY probe* ***5*** *(B) were counterstained with Evan's blue and observed on an inverted fluorescence microscope. The fluorescence was located in flat cells present in the lumen of the sinusoids.*

migrate to the liver and unload α-GalCer in some way. In this respect, IP macrophages were isolated from injected animals, washed, re-injected IV and found to induce depletion of liver NKT cells (data not shown).

Conclusion

The novel fluorescent α-galactoceramide **5** has been prepared from a versatile glycolipid precursor **7**. The synthesis of these original analogues of biologically active natural agelasphines or synthetic KRN7000 (**1**) was achieved by using a convergent approach. Elaboration of the key intermediates followed known procedures with D-*erythro*-sphingosine (**11**) and benzylgalactofluoride **8**. The novel spacer arm **10** was defined with a particular carbamate function, which allowed a final simultaneous olefinic reduction and debenzylation step. The molecules produced according to this novel and versatile synthetic route are about as active as the original KRN7000 (**1**) in the NKT-cell apoptosis test. This extends to the original BODIPY probe **5**. The biological assay was chosen because it is independent of the pattern of cytokines

released, which can be influenced by the nature of the fatty acyl chains.[5b, 20]

The fluorescent probe was used to visualise its own in vivo behaviour, which was assumed to mimic that of KRN7000 (**1**). A similar strategy has been widely used in the past with fluorescent derivatives of other natural glycolipids. Fluorescent probe **5** accumulated in antigen-presenting cells (APCs) regardless of the way it was administered. However, the type of APCs that captured the molecule differed with the method of administration. After IV injection, a significant concentration of fluorescent material was recovered in spleen and liver APCs, but not in other cell types, and particularly in spleen and liver leucocytes, which include NKT cells.

Experimental Section

Materials:

Mice: The C57BL/6 mice used in this study were 6 – 8 weeks old and were obtained from IFFA-Credo (L'Arbresle, France).

BODIPY: Obtained from Molecular Probes (Eugene, OR).

α-Galactosylceramide: The stock solution of α-GalCer (1 mg mL^{-1} in PBS 20% DMSO) was diluted in polysorbate 20 (300 μL, 0.25%; Sigma) in PBS. The vehicle control was used in all experiments. Wild-type C57BL/6 mice were injected (IP) with α-GalCer (1, 5 or 10 μg) for studying in vivo activity. Wild-type C57BL/6 mice were injected (IV) with α-GalCer **5** (50 μg) for imaging fluorescent cells.

Preparation of single-cell suspensions: Single-cell suspensions were prepared from liver and spleen. In the case of liver, total cells were re-suspended in 80% isotonic Percoll solution (Pharmacia, Uppsala, Sweden) and overlaid with 40% isotonic Percoll solution. Centrifugation for 30 min at 3000 rpm resulted in concentration of the mononuclear cells at the 40 – 80% interface. The collected cells were washed once with PBS supplemented with 2% fetal calf serum (FCS). Peritoneal cells were recovered by washing.

ABS and flow cytometry analyses: Cells were first incubated for 10 min with anti-Fc-g-III/II (Fcblock, 2.4G2) followed by a 30-min exposure to anti-TCRβ-APC (H57 – 597), NK1.1-PE (PK136) Abs (PharMingen). After further washes, the cells were re-suspended in PBS containing 2% FCS and analysed with a FACSCalibur BD Becton Dickinson instrument (San Jose, CA).

Adherent cells: the cells were further applied to culture dishes for 1.5 h at 37 °C in 5% CO_2. Adherent cells were studied by fluorescent microscopy (Zeiss, axiovert 135 TV).

Histology: Mice treated with α-GalCer were randomly selected for tissue harvesting after 1 h. Freshly isolated hepatic tissue was frozen in liquid nitrogen by using OCT embedding compound (Sakura, Zoeterwoude, Netherlands). Frozen sections (10 μm) were cut on a microcom HM 505 cryostat (Microcom Lab., Walldorf), fixed with PBS 4% paraformaldehyde and stained with Evan's blue, then photographed with an inverted microscope.

Instrumental analysis: Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) separations were performed on Waters 2690 and 996 apparatus (module separation and photodiiode array detector) on a Novapak C18 column. The solvent system used was 100% methanol then methanol/water (80:20). Optical rotations were measured at 20 °C with a Perkin – Elmer 141 automatic polarimeter. ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 or 400 and 75 MHz, respectively, with Bruker AC 300 and 400 spectrometers. ESI-MS spectra were recorded with a LCQDK Thermo-Finnigan Mass Spectrometer. IC-MS spectra were recorded on an AEI MS-9 spectrometer. High-resolution mass spectra were obtained with a Kratos MS 80RF spectrometer.

Carbamate 17: Benzylchloroformate (4.25 g, 25.0 mmol) was added to a stirred suspension of 11-aminoundecanoic acid (**16**; 10.0 g, 49.75 mmol) in dichloromethane (200 mL) at 20 °C. After stirring at reflux for 18 h, the solvent was evaporated and the resulting crude material was purified by flash column chromatography (cyclohexane/EtOAc, 1:1) to give **17** (2.5 g, 15%) as a white solid. ^{1}H NMR ($CDCl_3$): $\delta = 7.45 - 7.20$ (m, 5H; Ph), 5.05 (s, 1H; NH), 4.62 (s, 2H; OCH_2Ph), 3.12 (q, 2H, $J = 6.3$ Hz; H-11), 2.28 (t, 2H, $J = 7.5$ Hz; H-2), 1.6 – 1.15 (m, 16H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 178.1$ (CO_2H), 156.5 (NCO_2Bn), 136.5 (aromatic), 128.4 – 127.0 (aromatic), 66.7, 41.2, 34.0, 29.8, 29.3 – 29.0, 26.7, 24.7 (CH_2) ppm.

Activated ester 10: *Para*-nitrophenol (1 g, 7.46 mmol), dicyclohexylcarbodiimide (1.54, 7.46 mmol) and a catalytic amount of dimethylaminopyridine (30 mg) were successively added to a solution of benzylcarbamate **17** (2.5 g, 7.46 mmol) in dichloromethane (90 mL) at room temperature. After stirring for 24 h, the solvent was evaporated and the residue was purified by flash chromatography (cyclohexane/AcOEt, 7:3) to give pure compound **10** (1.6 g, 47%) as a white solid. ^{1}H NMR ($CDCl_3$): $\delta = 8.20$ (d, 2H, $J = 9.1$ Hz; aromatic), 7.30 (m, 5H; aromatic), 7.20 (d, 2H, $J = 9.1$ Hz; aromatic), 5.10 (s, 2H; OCH_2Ph), 4.95 (s, 1H; NH), 3.12 (q, 2H, $J = 6.3$ Hz; H-11), 2.55 (t, 2H, $J = 7.5$ Hz; H-2), 1.60 – 1.15 (m, 16H, $J = 6.3$ Hz; CH_2) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 171.2$ (CO_2H), 156.5 (NCO_2Bn), 155.4, 145.1, 136.7, 128.4, 127.9, 125.0, 122.3 (aromatic), 66.4, 41.0, 34.2, 29.8, 29.3 – 28.9, 26.6, 24.4 (CH_2) ppm.

Ceramide 9: A catalytic amount of dimethylaminopyridine (5 mg) was added to a solution of sphingosine (**11**; 110 mg, 0.37 mmol) and activated ester **10** (170 mg, 0.37 mmol) in THF (15 mL) at 20 °C. After stirring for 48 h, the solvent was evaporated and the residue purified by flash chromatography (CH_2Cl_2/MeOH, 95:5) to give pure **9** (173 mg, 76%) as a colourless wax. ^{1}H NMR ($CDCl_3$): $\delta = 7.35$ (m, 5H; aromatic), 6.35 (d, 1H, $J = 7.0$ Hz; NH), 5.75 (dt, 1H, $J = 15.3$; 6.6 Hz), 5.52 (dd, 1H, $J = 15.3$; 6.4 Hz), 5.11 (s, 2H), 4.80 (m, 1H; NH), 4.30 (m, 1H), 3.95 (m, 2H), 3.70 (m, 1H), 3.18 (q, 2H, $J = 6.5$ Hz), 3.10 (m, 1H; OH), 2.25 (t, 2H, $J = 7.5$ Hz), 2.07 (q, 2H, $J = 7.0$ Hz), 1.80 (m, 1H; OH), 1.65 – 1.20 (m, 30H; CH_2), 0.90 (t, 3H, $J = 6$ Hz; CH_3) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 174.1$ (NHCO), 156.5 (NCO_2Bn), 134.2, 128.9 – 128.6, 127.2 (aromatic), 66.7 (CH_2), 62.6, 54.7 (CH), 41.2, 36.9, 32.4, 32.0, 30.0, 29.7, 29.2, 26.7, 25.7, 22.8 (CH_2), 14.2 (CH_3) ppm; elemental analysis: calcd (%) for $C_{37}H_{64}N_2O_5$: C 72.04, H 10.46, N 4.54; found: C 71.91, H 10.67, N 4.38.

***α*-Galactosylceramide 18**: Silver perchlorate (60.5 mg, 0.30 mmol) and stannous chloride (55.4 mg, 0.30 mmol) were added to a stirred suspension of pre-activated powdered molecular sieves (350 mg) and galactosylfluoride **8** (80 mg, 0.15 mmol) in diethyl ether at 20 °C under argon. After 10 min, a solution of ceramide **9** (60 mg, 0.097 mmol) in a mixture of solvent (Et_2O/THF, 6:1) was introduced into the reaction mixture. Stirring was maintained for 4 h at room temperature and then the reaction was quenched with acetone (10 mL). After stirring for an additional 15 min, the solids were removed by filtration and the filter cake was washed with several portions of diethyl ether and ethyl acetate. Concentration of the solution gave an oily residue, which was purified by flash chromatography (cyclohexane/ethyl acetate, 8:2) to give pure compound **18** as a white wax (41 mg, 37%). $[\alpha]_D = +37$ ($c = 1.2$, CH_2Cl_2); ^{1}H NMR ($CDCl_3$): $\delta = 7.30$ (m, 5H; aromatic), 6.38 (d, 1H, $J = 8.1$ Hz; NH), 5.66 (dt, 1H, $J = 16.4$, 6.7 Hz), 5.42 (dd, 1H, $J = 15.4$, 5.4 Hz), 5.10 (s, 2H), 4.76 (s, 2H), 4.75 (d, 1H, $J = 3$ Hz), 4.85 and 4.20 (2d, 2H, $J = 11.1$ Hz),

4.55 and 4.90 (2 d, 2 H, $J = 11.4$ Hz), 4.45 and 4.35 (2 d, 2 H, $J = 11.7$ Hz), 4.13 (m, 1 H), 4.03 (dd, 1 H, $J = 9.9$, 3.6 Hz; H-11), 3.95 (m, 2 H), 3.88 – 3.75 (m, 3 H), 3.68 (dd, 1 H, $J = 10.5$, 3.8 Hz), 3.50 (m, 2 H), 3.17 (q, 2 H, $J = 6.6$ Hz), 2.12 (td, 2 H, $J = 7.4$, 2.4 Hz), 1.95 (m, 2 H), 1.60 – 1.20 (m, 42 H), 0.87 (t, 3 H, $J = 6.8$ Hz; Me) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 173.2$ (NHCO), 156.4 (NCO_2Bn), 138.5, 138.5 – 136.7 (aromatic) 133.0, 129.2 (olefinic), 128.5 – 127.4 (aromatic), 99.1, 79.3, 75.9, 74.8, 74.2 (CH), 74.5, 74.0, 73.4, 72.7 (CH_2), 69.8 (CH), 69.1, 68.7, 66.7 (CH_2), 52.8 (CH), 41.1, 36.7, 32.4, 31.9, 29.9 – 29.2, 26.7, 25.7, 22.8 (CH_2), 14.2 (CH_3) ppm.

Amino-α-galactosylceramide 7: A solution of compound **18** (30 mg, 0.046 mmol) in methanol (4 mL) and THF (2 mL) was treated with Pd/C (5 %, 30 mg) under hydrogen (1 bar) at room temperature for 3 days. The catalyst was removed by filtration through a celite bed and the filter cake was washed several times with small portions of chloroform. The combined filtrates were concentrated, and flash column chromatography (CH_2Cl_2/MeOH, 98:2 then 95:5) of the residue gave glycolipid **7** as a white wax (16 mg, 85 %). $[\alpha]_D = +60$ ($c = 0.75$, MeOH); ^{1}H NMR (CD_3OD): $\delta = 7.20$ (d, 1 H, $J = 8.0$ Hz; NH), 4.80 (d, 1 H, $J = 6.3$ Hz), 3.85 – 3.50 (m, 10 H), 2.70 (m, 2 H), 2.05 (m, 2 H), 1.61 – 1.11 (m, 44 H), 0.80 (t, 3 H, CH_3) ppm; ^{13}C NMR (CD_3OD): $\delta = 176.6$ (NHCO), 101.6, 72.8 – 70.7 (CH), 68.7, 63.2 (CH_2), 55.8 (CH), 48.3 (CH_2), 41.2 (CH), 37.7, 32.4 – 27.0 (CH_2), 24.2 (CH_3) ppm; FAB-MS: m/z: 647 $[M+H]^+$.

Amino-α-galactosylceramide-BODIPY 5: Compound **6** (5 mg, 0.011 mmol) in dimethylformamide (DMF; 0.2 mL) was added to a solution of α-galactosylceramide **7** (7 mg, 0.011 mmol) in DMF (0.5 mL) at 20 °C under an inert atmosphere. After stirring at room temperature for 36 h, concentration of the solution gave an orange residue. This residue was directly purified by HPLC (Novapak C18, MeOH then solvent gradient MeOH/$H_2$0, 8:2) to give a red solid (2.5 mg, 25 %), which was stored at -20 °C in the dark. ^{1}H NMR (CD_3OD): $\delta = 7.40$, 7.00, 6.30, 6.20 (4 s, 4 H), 3.90 – 3.40 (5 m, 8 H), 3.15 – 3.05 (m, 2 H), 2.60 (m, 2 H), 2.50 (s, 3 H), 2.30 (s, 3 H), 2.15 (m, 2 H), 1.60 – 1.20 (3 m, 44 H), 0.80 (t, 3 H, $J = 6$ Hz; CH_3) ppm; HR-MS (FAB): m/z calcd for $C_{49}H_{83}O_9N_4F_2BNa$: 943.6127 $[M+Na]^+$; found: 943.6107.

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