Asymmetric Synthesis of Water-Soluble Analogues of Galactosylceramide, an HIV-1 Receptor: New Tools to Study Virus – Glycolipid Interactions

Renaud Villard,[a] Djilali Hammache,[b] Guillaume Delapierre,[a] Frédéric Fotiadu,[a] Gérard Buono,*[a] and Jacques Fantini[b]

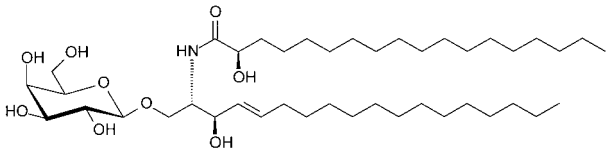
Galactosylceramide (GalCer) is a glycosphingolipid (GSL) receptor that allows HIV-1 infection of CD4-negative cells from neural and intestinal tissues. A water-soluble analogue of GalCer that features its polar head and the characteristic galactose – ceramide linkage but lacks the carbohydrate chains was prepared as a single enantiomer from (S)-serine. This analogue was not recognized in binding tests with the HIV-1 surface envelope glycoprotein gp120 in solution, which revealed the crucial importance of the ceramide alkyl chains. Two series of water-soluble GalCer analogues that contained either a hexanoic or a decanoic acyl unit and a saturated nine-carbon sphingosine moiety were designed by using molecular modeling results from natural GSLs and analogues with truncated alkyl chains. The longer chain compounds exhibit the characteristic fundamental conformation of GalCer. Seven analogues were prepared from Garner's aldehyde according to a straightforward and efficient asymmetric synthesis. All of these compounds proved to be water soluble but did not bind to gp120 in a solid-phase binding assay. These analogues were thus tested by using surface pressure measurements on a monomolecular film of GalCer, which served as a model of the plasma membrane. The incorporation of analogues very similar to GalCer into a GalCer monolayer prevented the insertion of gp120, whereas a structurally different derivative was not active. Based on these data, the molecular bases for recognition of GSLs by gp120 were elucidated. The essential importance of the GSL conformation in the primary interaction event and the crucial role of the alkyl chains of the ceramide moiety in the secondary interactions and the insertion process were clearly established.

KEYWORDS:

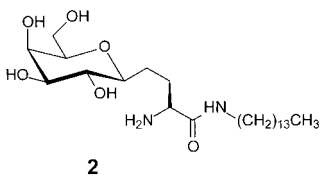
asymmetric synthesis · glycolipids · HIV · receptors

Introduction

Galactosylceramide (GalCer; **1**) is a monoglycosylated sphingolipid abundantly expressed in human neural and intestinal tissues.[1] It has been shown to be an alternative receptor that allows human immunodeficiency virus HIV-1 entry into cells that lack the classical HIV-1 receptor, surface protein CD4 ($CD4^-$ cells).[2] GalCer recognizes the V3 loop region of HIV-1 surface envelope glycoprotein gp120, which plays a key role in HIV-1 infection and pathogenesis.[3, 4, 5] The V3 domain can bind to a variety of anionic compounds, such as sulfated polysaccharides, heparin, and suramin, which are efficient inhibitors of HIV-1 infection in vitro.[6] GalCer is strictly insoluble in aqueous media thus some strategies have been developed towards water-soluble analogues of GalCer as potential inhibitors of HIV-1, such as compounds **2**[7] and **3**[8]. Fantini and co-workers demonstrated that recombinant HIV-1 gp120 specifically interacts with, and can penetrate into, a reconstituted membrane patch of galactosylceramide, which contains a 2-hydroxylated fatty acid, while ceramides, glucosylceramide, and nonhydroxylated GalCer are totally inactive.[9] These data prompted us to synthesize system-



GalCer **1**



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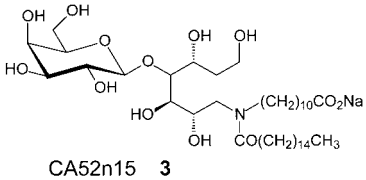

CA52n15 **3**

[a] *Prof. G. Buono, Dr. R. Villard, Dr. G. Delapierre, Dr. F. Fotiadu*
Ecole Nationale Supérieure de Synthèses de Procédés et d'Ingénierie
Chimiques d'Aix-Marseille, UMR 6516
Av. Escadrille Normandie-Niemen
13397 Marseille Cedex 20 (France)
Fax: (+33) 4-9128-2742
E-mail: buono@spi-chim.u-3mrs.fr

[b] *Dr. D. Hammache, Prof. J. Fantini*
Laboratoire de Biochimie et Biologie de la Nutrition
UPRESA 6033, Faculté des Sciences de St Jérôme
Av. Escadrille Normandie-Niemen
13397 Marseille Cedex 20 (France)

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atically modified analogues of GalCer to determine the minimum structure needed to bind specifically to gp120. We report here that such analogues allow one to identify the key factors that govern both the recognition and the fusion events between HIV-1 and the membranes of target cells.

Results and Discussion

Both the galactosyl moiety and the fatty acid 2-hydroxy group appeared to be necessary for binding to gp120, whereas galactose alone or simple alkylgalactosides are not recognized.[10] Thus, we first prepared analogue **4**, which features the polar head of GalCer and the characteristic galactose – ceramide linkage but lacks the hydrocarbon chains. This analogue was synthesized as a single enantiomer from the known protected serinol **5** (derived from (*S*)-serine),[11] by means of the 7-step transformation depicted in Scheme 1. Esterification of alcohol **5** with benzoyl chloride and pyridine then removal of the *tert*-butoxycarbonyl (Boc) and acetonide protection groups with aqueous 5 N HCl in 1,4-dioxane afforded enantiopure (*S*)-*O*-benzoyl serinol **6** in 48% yield. Acylation with acetoxyacetic acid under the usual conditions resulted in all cases in a mixture of ester and amide. We therefore set up the following alternative sequence: 1) protection of the amine with Boc_2O and NEt_3 in CH_2Cl_2 (95%), 2) glycosylation of the alcohol with pentaacetyl-D-galactose in the presence of $Et_2O \cdot BF_3$ in CH_2Cl_2, which lead exclusively to β-*O*-galactoside **7** (64%), 3) selective deprotection of the amine with phenol and $SiMe_3Cl$, 4) coupling with acetoxyacetic acid in the presence of *N*-[(dimethylamino)[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)oxy]methylene]-*N*-methylhexafluorophosphate (HdtU) and diisopropylethylamine (57%). Methanolysis of acetyl and benzoyl protecting groups with K_2CO_3 (67%) afforded analogue **4**.

Binding of the highly water soluble compound **4** to gp120 in solution was tested according to the method of Yahi et al.[6c] These tests showed that analogue **4** is not recognized by gp120. This result strongly suggests that the polar head of GalCer is not sufficient for binding and thus that the hydrocarbon chains of GalCer, responsible for its hydrophobicity, are of primary importance if the recognition process is to take place. Whether the lipophilic tail of the ceramide moiety is involved in interactions with gp120 or determines the biologically active conformation of the glycolipid remained to be established. We thus turned our attention to GalCer analogues with shortened alkyl chains. The challenge was to achieve sufficient water solubility whilst preserving most of the structure and thus the conformation of the natural glycosphingolipid (GSL). Since few data are available in the literature, we first performed a comprehensive conformational study of GalCer (**1**) and related GSLs.[12, 13] Systematic exploration of the conformation space of **1** revealed a very stable fundamental conformer among the 7.8×10^6 possibilities generated (Figure 1a). This lowest-energy structure agreed perfectly with reported X-ray studies on monoglycosylsphingolipids.[14] It exhibits the typical ceramide conformation in which the two alkyl chains of the sphingosine and fatty acid groups are parallel to each other and perpendicular to the plane of the amide function.[15] A strong hydrogen bond between the amide NH group and the glycosidic oxygen forces the galactose head group to adopt a parallel orientation relative to the cell surface in which the ceramide would be anchored.[14c] It is remarkable that the conformation we found for an isolated GalCer molecule was so close to structures observed in crystals that are considered to mimic the conformations adopted by GSLs within membranes. This reveals that the characteristic conformational arrangement of GSLs results from intramolecular forces rather than intermolecular interactions in the membrane environment.

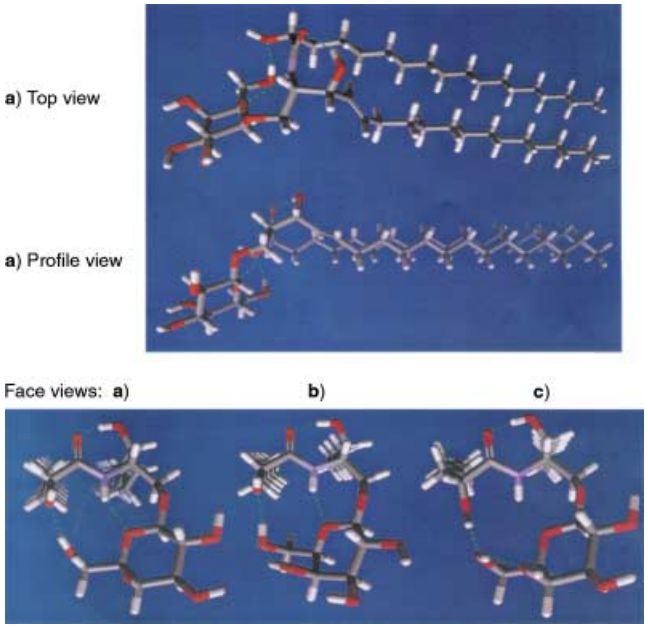
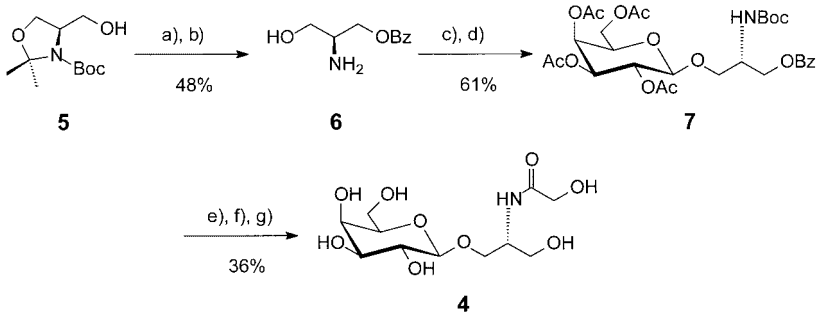


Figure 1. *Molecular views of the characteristic fundamental conformations of (a) natural GalCer (top, profile, and face view), (b) an analogue with a nine-carbon-atom sphingosine chain and ten-carbon-atom acyl unit (face view), and (c) an analogue with a saturated nine-carbon-atom sphingosine chain and a six-carbon-atom acyl unit. Hydrogen bonds are shown by dotted lines.*

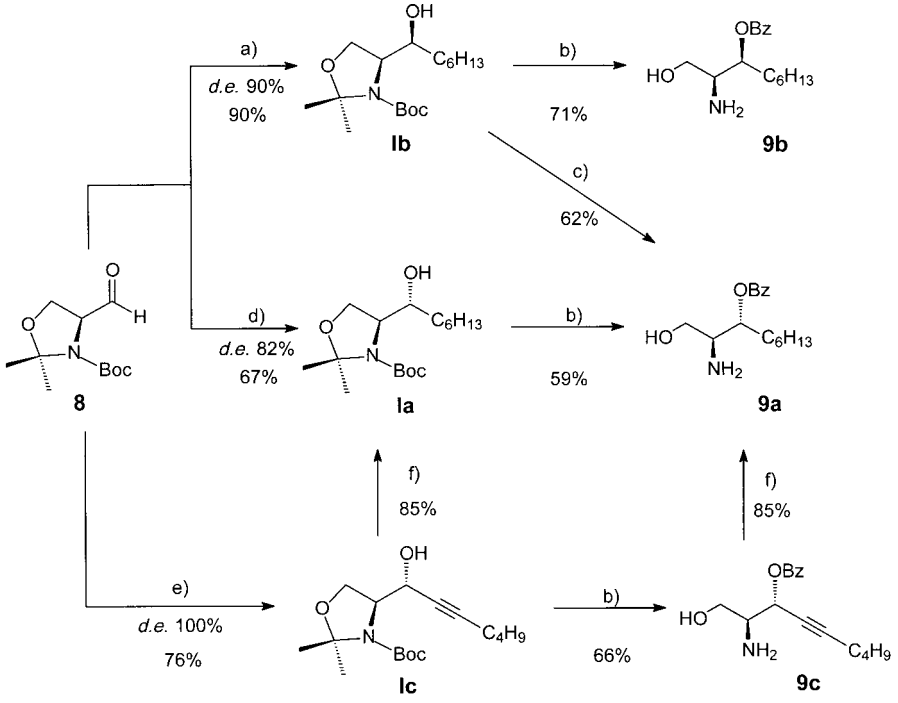


Scheme 1. *Conditions: a) BzCl, pyridine, RT, 12 h; b) HCl (5 N), dioxane, reflux, 1 h; c) Boc_2O, NEt_3, CH_2Cl_2, RT, 4 h; d) penta-O-acetyl-β-D-galactose, $Et_2O \cdot BF_3$, CH_2Cl_2, RT, 2 h; e) Me_3SiCl, PhOH, RT, 2 h; f) $AcOCH_2CO_2H$, HDtU, diisopropylethylamine, RT, 12 h; g) K_2CO_3, MeOH, RT, 3 h. Bz = benzoyl.*

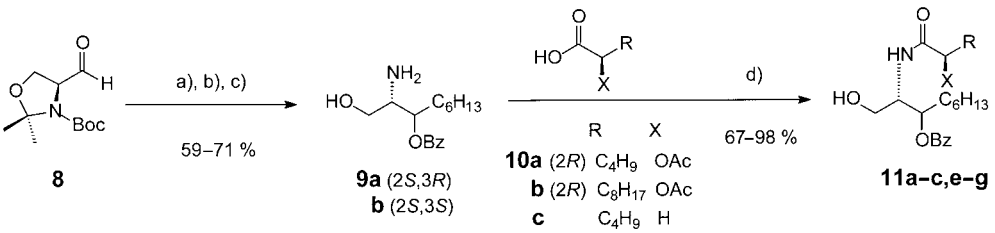
Similar calculations were carried out with different alkyl chain lengths and in the presence

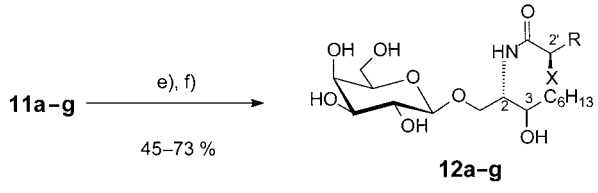
and absence of the double bond and the hydroxy group on the sphingosine and acid chains, respectively. We established that models with saturated sphingosine and acid moieties longer than nine carbon atoms exhibited lowest-energy structures very similar to that of GalCer (Figure 1 b), whereas those with shorter chains (like **4**) had completely different stable conformations (Figure 1 c). Based on these results, we chose to prepare two series of analogues with slightly different lipophilicity, which should adopt the characteristic conformation of GalCer. These analogues derive from a saturated sphingosine (sphinganine) fragment with nine carbon atoms, that is, half the natural chain length (C18). We considered either a hexanoic or a decanoic acid moiety, chain lengths which span the observed critical length of nine carbon atoms. Calculations predicted that the second series (with a decanoic acid moeity) would exhibit GalCer-like fundamental conformations, whereas the first series (hexanoic acid moeity) has different lowest-energy structures but could also adopt conformations similar to GalCer that span energies only a few kiloJoule higher in their conformational spectra.

We developed a straightforward synthetic methodology based on acylation of the sphingosine moiety at the nitrogen atom first, followed by glycosylation of the ceramide obtained, and optimized the protection – deprotection strategy.[16] In an earlier study, we described the highly diastereoselective addition of dihexylzinc and hexylmagnesium bromide to Garner's chiral aldehyde **8**,[17] which is derived from (*S*)-serine.[18] Subsequent benzoylation and removal of the acetonide and Boc protecting groups by acid hydrolysis allowed us to obtain the diastereomers of benzoyl dihydrosphingosine **9a** (with natural stereochemistry) and **9b** in 59 and 71% yield, respectively, from intermediate compounds **1a – c** (Schemes 2 and 3).[19, 20] Each compound was used to prepare a series of GSL analogues to evaluate the influence of the absolute configuration of the sphingosine 3-hydroxy group on recognition.[21] Protected aminodiols **9a**,**b** were selectively acylated at the nitrogen atom by either 2(*R*)-acetoxyhexanoic acid **10a**, 2(*R*)-acetoxydecanoic acid **10b**, or hexanoic acid **10c**, by using coupling reagents commonly used in peptide synthesis.[16d, 22] In the presence of 1-benzotriazolyloxytris(dimethylamino)phos-



Scheme 3. *Conditions: a) $C_6H_{13}MgBr$, Et_2O, room temperature (RT), 3 h; b) (i) BzCl, pyridine, RT, 12 h; (ii) HCl (5 N), dioxane, 100 °C, 1 h; c) (i) diethylazodicarboxylate, PPh_3, $PhCO_2H$, THF, RT, 3 h; (ii) HCl (5 N), dioxane, 100 °C, 1 h; d) $(C_6H_{12})_2Zn$,* N,N*-dibutylethanolamine, toluene, RT, 2 h; e) 1-hexynelithium, THF, RT; 3 h; f) H_2, PtO_2 cat., AcOEt, RT, 2 h.*





	11 or 12	R	X
(2S,3R,2'R)	a	C_4H_9	OH
(2S,3R,2'R)	b	C_8H_{17}	OH
(2S,3R)	c	C_4H_9	H
(2S,3S)	d	C_8H_{17}	H
(2S,3S,2'R)	e	C_4H_9	OH
(2S,3S,2'R)	f	C_8H_{17}	OH
(2S,3S)	g	C_4H_9	H

Scheme 2. *Conditions: a) $(C_6H_{12})_2Zn$,* N,N*-dibutylethanolamine, toluene, RT, 2 h; or $C_6H_{13}MgBr$, Et_2O, RT, 3 h; b) BzCl, toluene/pyridine, RT, 12 h; c) HCl (5 N), dioxane, 100 °C, 1 h; d) BOP or HDtU, diisopropylethylamine, CH_2Cl_2, RT, 24 h; e) penta-*O*-acetyl-β-D-galactose, $Et_2O \cdot BF_3$, CH_2Cl_2, RT, 2 h; f) K_2CO_3, MeOH, RT, 3 h.*

phonium hexafluorophosphate (BOP), or alternatively HDtU, and diisopropylethylamine in CH_2Cl_2, amides **11a–c,e–g** were obtained in 67–98% yield. Surprisingly, decanoic acid only underwent esterification under these conditions and preparation of **11d** required a three-step protocol that involved initial protection of the primary hydroxy group of **9a** as a trimethylsilyl (TMS) ether. Condensation of *n*-decanoyl chloride followed by removal of the silyl protecting group under mildly acidic conditions, produced by using a stoichiometric amount of TMSCl to generate HCl in MeOH, provided amide **11d** in 63% overall yield. Glycosylation of ceramide analogues **11a–g** with penta-*O*-acetyl-β-D-galactopyranose in the presence of $BF_3 \cdot OEt_2$ yielded exclusively the expected β-*O*-glycosides intermediates **II** (35–86%, see the Experimental Section for details).[23, 24] Finally, subsequent removal of the benzoyl and acetyl protecting groups by methanolysis under mild conditions with K_2CO_3 in MeOH afforded the target analogues **12a–g** in 45–73% yield (Scheme 2). All the target analogues **12a–g** proved to be water soluble up to 10^{-4} mol L^{-1}, which allowed binding tests to be run with gp120.

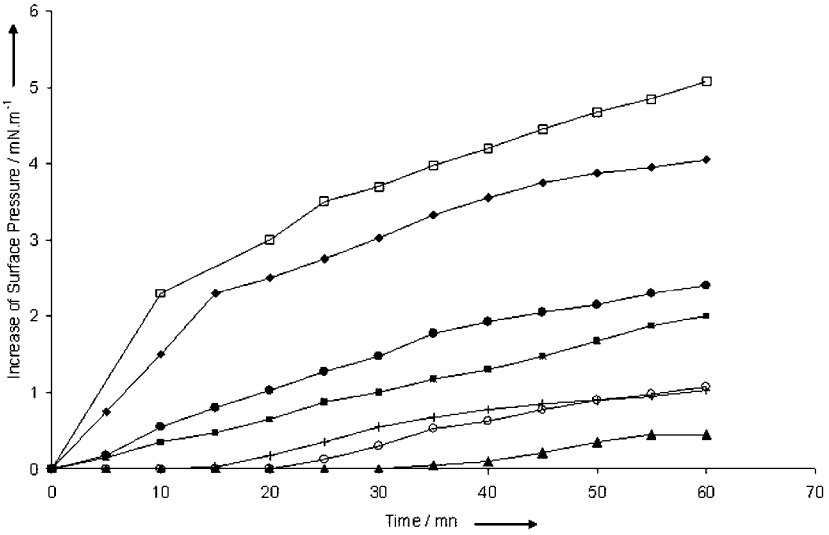


Figure 2. *Effect of GalCer analogues on GalCer–gp120 interactions studied by using a monomolecular film of GalCer. The surface pressure increase (Δ) induced by addition of gp120 to a GalCer monolayer is shown. Analogues that interfere with this interaction (for example, **12a**) induce a decrease in Δ. In contrast, analogues such as **12c**, which does not compete with natural GalCer for gp120 binding, do not affect the Δ value. (◆) GalCer, (▲) **12a**, (○) **12b**, (□) **12c**, (■) **12d**, (●) **12e**, (+) **12f**.*

The synthetic analogues were first evaluated for their ability to recognize the HIV-1 surface envelope glycoprotein gp120 in a solid-phase binding assay.[25] In marked contrast to the results obtained with deoxylactitol derivatives,[8] the present series of water-soluble analogues did not bind to gp120 when added in solution to the viral glycoprotein, which was bound on microtiter polystyrene plates. In this respect, the synthetic analogues behave exactly like 3′-sulfoGalCer, the natural water-soluble sulfated GalCer derivative, which does not bind to gp120 in the same assay. However, when sulfatide was incorporated into a monomolecular film of GalCer, it was able to block the insertion of gp120 into the GalCer film.[26] This observation prompted us to test the present series of GalCer analogues under similar conditions by using a monomolecular film of glycosphingolipids at the air–water interface as a model for the GSL patches of the plasma membrane.[27] As shown in Figure 2, the incorporation of analogue **12a** into a GalCer monolayer completely abrogated the insertion of gp120 ($\Delta\Pi < 1$ mN m^{-1}). In contrast, the non-hydroxylated derivative **12c** was not active and its incorporation in the monolayer did not impair gp120 insertion. This result is in perfect agreement with previous data obtained for natural hydroxylated and nonhydroxylated forms of GalCer and validates the methodology presented.[9] The other derivatives could be classified with respect to their activity in the monolayer assay to give an activity ranking of **12f** = **12b** > **12d** > **12e**. Finally, the **12g** analogue was totally inactive in the monolayer assay (not shown).

As expected, the most active analogues **12a** and **12b** feature exactly the same polar head as natural GalCer (same functionality and same stereochemistry), with the shorter alkyl chain compound **12a** even more active than the longer one. Thus, the molecular recognition process with gp120 does occur in a very specific way and involves both galactose (as previously established) and the polar functional head of the ceramide moiety. These primary interactions should occur at the interface of the monolayer and should not affect its surface pressure. In a second step, lipophilic interactions between gp120 and the hydrocarbon chains of the ceramide moiety may lead to partial insertion of the protein inside the GSL monolayer.[26] We clearly show that analogues possessing shortened chains do not allow such secondary interactions to occur and either abrogate (**12a**) or strongly reduce further insertion (**12b**), depending on the length of the remaining chains.

The absolute configuration of the carbon 3 atom of the sphingosine unit does not seem to play any role in the initial recognition process since **12b** and **12f** had identical inhibitory effects. Both were proved to adopt similar, GalCer-like, stable conformations. In contrast, different behaviors were observed for the analogues with a short acyl chain and opposite absolute configuration at carbon 3 (compare **12a** and **12e**). In fact, we found that characteristic GalCer-like conformations lay at higher energies in the conformational spectrum of compound **12e** compared to that of compound **12a**. Thus, **12a** can easily adopt the conformation required for the initial interaction with gp120 to occur, whereas **12e** mainly exists as different conformers that are not recognized.

It is worth noting that the molecular modeling study clearly established that the analogues with short acyl chains, **12c** and **12g**, did not present any conformation similar to GalCer and thus could not be recognized by gp120 in the primary step. Furthermore, because these compounds present molecular shapes that are very different from those of GSLs and have high water solubility, they should not insert into a GalCer monolayer as a GSL mimic. Such behavior was indeed observed for both

compounds. Analogue **12g** did not significantly insert into a pure GalCer monomolecular film, whereas **12c** did. However, **12c** allowed gp120 insertion up to a higher surface pressure than pure GalCer. This strongly suggests that compound **12c** does not behave like a natural GSL and greatly disturbs the molecular arrangement of the monolayer, contrary to the other analogues.

The essential importance of conformational factors in the molecular recognition phenomenon was further confirmed by the fact that **12d** exhibited low but significant activity compared to the other non-α-hydroxylated analogues **12c** and **12g**. As explained above, these non-α-hydroxylated analogues cannot adopt the required conformation to interact with gp120. This was not the case with compound **12d**, which exhibited a conformation very similar to those of **12f** and GalCer despite the lack of a hydroxy group on its acyl chain. Consequently, much weaker interactions with the viral glycoprotein might occur in the absence of the α-OH group, which lead to a low remaining activity.

Taken together, these data allowed identification of the α-OH group on the fatty acid unit as a critical active site in the GalCer molecule and strongly supported the hypothesis that this site is directly involved in gp120 binding in the primary interaction step. In contrast, the *trans* double bond of sphingosine in the ceramide moiety of natural GalCer does not seem to play any role in this initial event. In fact, saturated analogues we prepared were unambiguously recognized by HIV-1 gp120, despite their lack of this double bond.[28]

In conclusion, we have developed an efficient asymmetric synthesis of very similar analogues of natural GalCer. These analogues were used to clearly establish the molecular basis for the selective recognition process between HIV-1 surface glycoprotein and GSL analogues within a GalCer monolayer. We provided evidence that this process involves conformations similar to the fundamental conformer of GalCer and demonstrated that the alkyl chains of the ceramide moiety are essential to the gp120 insertion into the monolayer. These chains are mainly responsible for the active conformation of GalCer which is necessary for the primary interaction with gp120. The chains also directly control the insertion process of the viral glycoprotein into the GSL monolayer through secondary lipophilic interactions. These results shed some light on the molecular mechanism of HIV-1 recognition and entry into cells that express GalCer. From a more general point of view, the optimized synthetic pathway we describe will allow numerous other structural modifications and further structure – activity relationship studies. The methodology and synthetic analogues we disclose here provide novel and valuable tools for the study of virus – glycoplipid interactions.

Experimental Section

General: Toluene was distilled over calcium hydride. Tetrahydrofuran (THF) and diethyl oxide were distilled from sodium and benzophenone (indicator). ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC200 spectrometer at 200 and 50.36 MHz, respectively, with the solvent as internal standard. Infrared spectra were recorded as thin films for liquids and KBr disks for solids (Perkin-Elmer 298 IRFT). Optical rotation measurements were taken on a Perkin-Elmer 341 polarimeter. TLC was carried out on silica gel 60 F254 (SDS, Peypin, France) with detection by UV light or phosphomolybdic acid. Mass spectroscopy analyses were performed at the Service de Mesure Physique, Faculté des Sciences de Montpellier (34), France by using the FAB technique with a gt matrix.

2(*S*)-amino-3-hydroxypropyl benzoate (6) from protected serinol 5:

A method similar to the typical procedure for synthesis of **9a – c** from **Ia – c** was used (see below). Yld: 48%; colorless solid, mp: 121 °C; $R_f = 0.2$ (AcOEt); $[\alpha]_D^{20} = +3.1$ ($c = 3.6$, MeOH); IR (KBr): $\tilde{\nu} = 3520$ (OH, NH), 3082 ($C_{ar}H$), 2978 ($C_{al}H$), 1669 (C=O) cm^{-1}; ^{1}H NMR (CD_3OD): $\delta =$ 3.77 (m, 4H), 4.05 (m, 1H), 7.45 (m, 5H), 7.90 (m, 2H) ppm; ^{13}C NMR (CD_3OD): $\delta = 47.5$, 54.2, 63.2, 127.2, 128.5, 131.7, 142.3, 175.4 ppm; $C_{10}H_{13}NO_3$ (195.21).

Protected galactoside 7:

Boc-protected derivative of **6**: Triethylamine (3.0 mmol) was slowly added to a stirred solution of **6** (1.0 mmol) and di-*tert*-butyl dicarbonate (1.2 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred for 3 h at 25 °C and then washed successively with aqueous solutions of $KHSO_3$ (1N, 10 mL), $NaHCO_3$ (50%, 10 mL), and citric acid (10%, 10 mL) and brine (10 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. After silica-gel column chromatography (petroleum ether/AcOEt, 5:1), we obtained the desired compound (0.27 g, 95%) as a colorless solid. Mp: 88 °C; $R_f = 0.6$ (petroleum ether/AcOEt, 5:1). $[\alpha]_D^{20} = +4.30$ ($c = 1.0$, $CHCl_3$); IR (KBr): $\tilde{\nu} =$ (OH, NH), 2985 ($C_{ar}H$), 2917 ($C_{al}H$), 2862 ($C_{al}H$), 1743 (C=O), 1678 (C=O) 3406 cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 1.36$ (s, 9H), 4.03 – 4.32 (m, 6H), 5.79 (d, $^3J = 12.1$ Hz, 1H), 7.32 (m, 3H), 7.66 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 28.3$, 51.0, 65.1, 67.0, 87.1, 127.1, 128.4, 131.5, 146.7, 153.4, 167.3 ppm; $C_{15}H_{21}NO_5$: 295.33.

For the second step required to prepare **7** from the previous compound, see the typical procedure for protected analogues of glycosphingolipids **IIa – g**. Yld: 64%; brown oil; $R_f = 0.3$ (AcOEt/petroleum ether, 1:1); $[\alpha]_D^{20} = +25.4$ ($c = 0.6$, $CHCl_3$); IR (film): $\tilde{\nu} =$ 3436 (NH), 3070 ($C_{ar}H$), 2978 ($C_{al}H$), 2907 ($C_{al}H$), 1770 – 1740 (C=O), 1669 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 1.20$ (s, 9H), 1.84 (m, 14H), 3.91 – 4.12 (m, 5H), 5.01 – 5.12 (m, 5H), 6.53 (m, 1H), 7.32 (m, 3H), 7.72 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 20.3$, 20.5, 20.7, 27.1, 53.1, 61.0, 65.7, 66.2, 67.2, 68.1, 69.4, 70.8, 88.2, 100.8, 126.7, 128.3, 131.6, 140.3, 158.0, 166.2, 169.2, 169.8, 170.2 ppm; $C_{29}H_{39}NO_{14}$: 625.62.

Analogue 4:

Protected analogue **4**: TMSCl (0.90 mmol) was slowly added to a stirred solution of **7** (0.30 mmol) and phenol (0.90 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred for 2 h at 25 °C then washed successively with an aqueous solution of $NaHCO_3$ (50%, 10 mL) and brine (10 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. This residue was dissolved in CH_2Cl_2 (10 mL). 2-acetoxyethanoic acid (0.33 mmol) and HDtU (0.33 mmol) were added and the mixture was cooled to 0 °C. Diisopropylethylamine (0.6 mmol) was slowly added and the solution was stirred for 12 h at 25 °C, diluted with AcOEt (15 mL), and washed successively with 1N HCl (2×10 mL), 1N $NaHCO_3$ (2×10 mL) and brine (10 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. After silica-gel column chromatography (petroleum ether/AcOEt, 1:1), we obtained the desired compound (0.10 g, 57%) as a brown oil. $R_f = 0.2$ (petroleum ether/AcOEt, 1:1); $[\alpha]_D^{20} = +1.30$ ($c = 1.0$, $CHCl_3$); IR (KBr): $\tilde{\nu} = 3350$ (OH, NH), 3080 ($C_{ar}H$), 2992 ($C_{al}H$), 1750 – 1740 (C=O), 1661 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 1.84$ (m, 17H), 3.73 – 3.88 (m, 5H), 4.38 – 4.52 (m, 5H), 6.17 (m, 1H), 7.28 (m, 3H), 7.72 (m,

2H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 20.3$, 20.5, 20.7, 52.9, 61.2, 65.3, 66.0, 67.1, 68.1, 69.2, 70.5, 100.1, 126.4, 128.1, 131.5, 139.8, 161.1, 168.3, 168.6, 169.5, 170.1 ppm; $C_{28}H_{35}NO_{15}$: 625.58.

For the last step in the synthesis of compound **4**, see the typical procedure for unprotected analogues of glycosphingolipids **12a–g**. Yld: 64%; mp: 150 °C; IR (KBr): $\tilde{\nu} = 3450$ (OH, NH), 2973 ($C_{al}H$), 2901 ($C_{al}H$), 1663 (C=O) cm^{-1}; ^{1}H NMR (D_2O): $\delta = 0.90$ (m, 6H), 1.20–1.45 (m, 24H), 3.40–3.60 (m, 3H), 3.90–4.30 (m, 9H), 7.19 (m, 1H) ppm; MS: *m/z* (%): 312 (0) $[MH]^+$, 115 (60) $[C_4H_5NO_3]^+$, 73 (100) $[C_3H_7NO]^+$, 44 (55) $[C_2NO]^+$; $C_{11}H_{21}NO_9$ (311.28).

Intermediate compounds 1a and 1b:

Method 1: Hexylmagnesium bromide, prepared from 1-bromohexane (3.24 g, 19.6 mmol) and magnesium (0.48 g, 19.6 mmol) in diethyl ether, was added dropwise under an inert atmosphere to a stirred solution of 4(*S*)-formyl-2,2-dimethyl-1,3-oxazolidine tertiobutylcarboxylate (aldehyde **8**; 1.5 g, 6.5 mmol) at −20 °C. After stirring for 3 h at 25 °C, a saturated aqueous solution of ammonium chloride (50 mL) was added. The mixture was extracted with AcOEt (3 × 30 mL). Organic layers were washed with brine (50 mL), dried over Na_2SO_4 and concentrated in vacuo. After silica-gel column chromatography (petroleum ether/AcOEt, 1:1), two diastereomers **1a** (0.092 g, 5%) and **1b** (1.75 g, 85%) were obtained in a 95:5 ratio.

Method 2: Under an inert atmosphere, *N*,*N*-dibutylethanolamine (0.55 g, 3.2 mmol) was added dropwise to a stirred ice-cooled 5 N solution of dihexylzinc in toluene (0.78 mL, 3.9 mmol).[18] After stirring for 0.5 h at 0 °C, $ZnCl_2$ (0.18 g, 1.3 mmol) and aldehyde **8** (0.30 g, 1.3 mmol) were added. Stirring was continued for 2 h at 25 °C then a saturated solution of ammonium chloride (20 mL) was added. The mixture was extracted with AcOEt (50 mL). The organic layer was washed with brine (20 mL), dried over Na_2SO_4 and concentrated in vacuo. After silica-gel column chromatography (petroleum ether/AcOEt, 1:1), two diastereomers **1a** (0.25 g, 61%) and **1b** (0.025 g, 6%) were obtained in a 9:91 ratio.

Intermediate compound **1a**: Orange oil; $R_f = 0.3$ (AcOEt/petroleum ether, 1:3); $[\alpha]_D^{20} = -38$ ($c = 1.17$, $CHCl_3$); IR (film): $\tilde{\nu} = 3500$ (OH), 2950 ($C_{al}H$), 2894 ($C_{al}H$), 2820 ($C_{al}H$), 1710 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 0.90$ (t, $^3J = 6.6$ Hz, 3H), 1.28–1.60 (m, 25H), 3.61–4.03 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 14.1$, 22.6, 25.4, 26.4, 28.2, 29.2, 29.6, 31.8, 61.9, 63.0, 64.8, 81.0, 93.9, 154.3 ppm; elemental analysis: calcd for $C_{17}H_{33}NO_4$ (315.45): C 64.7, H 10.5, N 4.4; found: C 64.5, H 10.3, N 4.5.

Intermediate compound **1b**: Yellow oil; $R_f = 0.4$ (AcOEt/petroleum ether, 1:3); $[\alpha]_D^{20} = -17$ ($c = 2.23$, $CHCl_3$); IR (film): $\tilde{\nu} = 3500$ (OH), 2950 ($C_{al}H$), 2894 ($C_{al}H$), 2820 ($C_{al}H$), 1716(C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta =$ 0.90 (t, $^3J = 6.6$ Hz, 3H), 1.28–1.60 (m, 25H), 3.55–4.10 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 14.0$, 22.6, 25.4, 26.4, 28.4, 29.3, 29.6, 31.9, 61.9, 62.8, 64.8, 81.1, 94.0, 156.0 ppm; elemental analysis: calcd for $C_{17}H_{33}NO_4$ (315.45): C 64.7, H 10.5, N 4.4; found: C 64.2, H 10.3, N 4.6.

Intermediate compound 1c:

Under an inert atmosphere, a solution of *n*-butyllithium in toluene (1.6 M, 12 mL, 19.65 mmol) was added dropwise to a stirred solution of 1-hexyne (1.6 g, 19.65 mmol) in THF (20 mL) at −20 °C. Stirring was continued for 2 h at −20 °C and then the solution was cooled to −78 °C. A solution of **8** (3.0 g, 13.1 mmol) in THF (5 mL) was added dropwise and stirring was continued for 1 h at −78 °C and for 2 h at −20 °C. A saturated solution of ammonium chloride (30 mL) was added, the mixture was concentrated in vacuo and diethyl oxide was added (100 mL). The organic layer was washed with aqueous HCl (1 N; 50 mL) and brine (50 mL), dried over $MgSO_4$, and concentrated in vacuo. After a silica-gel column chromatography (CH_2Cl_2), **1c** (3.1 g, 76%) was obtained as a pale yellow oil; $R_f = 0.3$ (CH_2Cl_2); $[\alpha]_D^{20} =$ −48.8 ($c = 1.8$, $CHCl_3$); IR (film): $\tilde{\nu} = 3441$ (OH), 2990 ($C_{al}H$), 2941 ($C_{al}H$), 2872 ($C_{al}H$), 2308 (C≡C), 1706 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 0.89$ (t, $^3J = 7.2$ Hz, 3H), 1.32–1.58 (m, 19H), 2.19 (td, $^3J = 6.8$, 1.8 Hz, 2H), 3.93 (br s, 1H), 4.08 (t, $^3J = 3.0$ Hz, 2H), 4.55 (br s, 1H), 4.70 (br s, 1H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 13.5$, 18.3, 21.8, 25.7, 28.7, 30.5, 62.7, 64.0, 65.0, 77.9, 80.8, 86.2, 94.7, 153.7 ppm; elemental analysis: calcd for $C_{17}H_{29}NO_4$ (311.45): C 65.6, H 9.4, N 4.5; found: C 65.4, H 9.5, N 4.5

Saturated compound 1a from alkyne 1c:

PtO_2 (7 mg, 0.029 mmol) was added to a solution of **1c** (200 mg, 0.642 mmol) in AcOEt (5 mL). The mixture was stirred for 2 h at 25 °C under a hydrogen atmosphere, filtered, and concentrated in vacuo. After silica-gel column chromatography (AcOEt), **1a** (171 mg, 85%) was obtained as a pale yellow solid. See above for analytical data.

Protected sphingosine analogues 9a, 9b, and 9c:

Typical procedure: Benzoyl chloride (20 mmol) was slowly added to a stirred solution of alcohol **1a**, **1b**, or **1c** (10 mmol) and dimethylaminopyridine (1 mmol) in a mixture of toluene/pyridine (4:1, 20 mL). The mixture was stirred overnight at 25 °C and then filtered. The solid was washed with toluene (3 × 5 mL) and the organic layers were washed with brine (2 × 15 mL), dried over $MgSO_4$, and concentrated in vacuo. The crude product was diluted in a mixture of dioxane (110 mL) and 1 N HCl (60 mL), refluxed for 1 h, and cooled to 25 °C. A solution of aqueous 2 N NaOH was added until pH 10 was reached. The mixture was extracted with CH_2Cl_2 (3 × 250 mL). The organic layers were washed with brine (250 mL), dried over $MgSO_4$, and concentrated in vacuo. After silica-gel column chromatography (petrolueum ether/AcOEt, 1:1), compound **9a**, **9b**, or **9c** was obtained as a pale yellow solid.

Sample data for **9a**: Yld: 71%; mp: 74 °C; $R_f = 0.4$ (petroleum ether/AcOEt, 1:3); $[\alpha]_D^{20} = +14.9$ ($c = 1.0$, $CHCl_3$); IR (KBr): $\tilde{\nu} = 3400$ (OH, NH), 3050–3010 ($C_{ar}H$), 2993 ($C_{al}H$), 2877 ($C_{al}H$), 1640 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 0.88–0.91$ (t, $^3J = 6.6$ Hz, 3H), 1.25–1.53 (m, 10H), 3.22 (m, 1H), 3.90–3.92 (d, $^3J = 4.2$ Hz, 2H), 4.04–4.17 (m, 2H), 7.01 (d, $^3J = 8.2$ Hz, 2H), 7.47 (m, 3H), 7.82 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): $\delta =$ 14.0, 34.3, 31.7, 29.1, 25.6, 22.6, 54.0, 64.4, 72.0, 127.0, 128.5, 131.6, 134.0, 168.3 ppm; MS: *m/z* (%): 280 (100) $[MH]^+$, 262 (25) $[MH - H_2O]^+$, 105 (50) $[PhCO]^+$. $C_{16}H_{25}NO_3$: 279.37.

Analytical data for compounds **9b** and **9c** are provided in the Supporting Information.

Alternative procedures for **9a**:

From **9c**: PtO_2 (7 mg, 0.029 mmol) was added to a solution of **9c** (200 mg, 0.726 mmol) in AcOEt (5 mL). The mixture was stirred for 2 h at 25 °C under a hydrogen atmosphere then filtered and concentrated in vacuo. After silica-gel column chromatography (AcOEt), **9a** (171 mg, 85%) was obtained as a pale yellow solid. See above for analytical data.

From **1b**: Under an inert atmosphere, a solution of **1b** (1.6 g, 5 mmol), triphenylphosphine (2.6 g, 10 mmol), and benzoic acid (1.2 g, 10 mmol) in diethyl oxide (100 mL) was stirred for 0.25 h at 25 °C. Diethylazodicarboxylate (1.8 g, 10 mmol) was slowly added. After stirring for 2 h at 25 °C, the mixture was concentrated in vacuo and diluted in a mixture of dioxane (50 mL) and HCl (5 N; 50 mL), refluxed for 1 h, and cooled in ice. An aqueous solution of 1 N NaOH was slowly added until the pH 1 was reached. The aqueous solution was extracted by CH_2Cl_2 (3 × 50 mL). Organic layers were washed with brine (25 mL), dried over Na_2SO_4 and concentrated in vacuo. After silica-gel column chromatography (petroleum ether/AcOEt, 1:1), **9a** (0.9 g, 62%) was obtained as a colorless solid. See above for analytical data.

2(*R*)-acetoxyacids ((*R*)-10b):

Typical procedure for 2-bromoacids: A solution of hexanoic (or decanoic) acid (0.1 mol), bromine (0.11 mol), and phosphorus

trichloride (0.01 mol) was refluxed until the solution became clear and then distilled under reduced pressure to obtain the 2-bromoacid as a clear oil.

2-bromohexanoic acid: Yld: 92%; $bp_{0.1}$: 69 °C; IR (film): $\tilde{\nu}$ = 3052 (OH), 2988 ($C_{al}H$), 2926 ($C_{al}H$), 2867 ($C_{al}H$), 1717 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 1.00 (t, ^3J = 7.1 Hz, 3H), 1.45 (m, 4H), 2.03 (m, 2H), 4.21 (t, ^3J = 7.2 Hz, 1H), 12.26 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ = 13.6, 21.9, 29.2, 34.2, 45.3, 176.3; $C_6H_{11}BrO_2$: 195.05.

2-bromodecanoic acid: Yld: 84%; $bp_{0.05}$: 118 – 120 °C; IR (film): $\tilde{\nu}$ = 3027 (OH), 2934 ($C_{al}H$), 2850 ($C_{al}H$), 1717 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0.86 (t, ^3J = 7.0 Hz, 3H), 1.25 (m, 12H), 2.07 (m, 2H), 4.21 (t, ^3J = 7.2 Hz, 1H), 12.10 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ = 14.1, 22.5, 27.1, 28.7, 29.1, 29.2, 31.7, 34.6, 45.3, 176.3 ppm; $C_{10}H_{19}BrO_2$: 251.16.

Typical procedure for 2-hydroxyacids: A solution of 2-bromoacid (0.05 mol) in aqueous NaOH (0.5 N; 200 mL) was refluxed for 3 h and then ice-cooled. An aqueous solution of HCl (3 N) was slowly added until pH 2 was reached. This aqueous layer was extracted with AcOEt (3 × 80 mL). The organic layers were dried over $MgSO_4$ and concentrated in vacuo. Recrystallization of the residue from petroleum ether/diethyl oxide (20:1) afforded **7** as a white solid.

2-hydroxyhexanoic acid: Yld: 93%; mp: 60 °C; IR (KBr): $\tilde{\nu}$ = 3300 (OH), 2950 ($C_{al}H$), 2872 ($C_{al}H$), 2818 ($C_{al}H$), 1723 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0.99 (t, ^3J = 6.0 Hz, 3H), 1.20 – 1.65 (m, 6H), 1.85 (br s, 1H), 4.36 (t, ^3J = 7.8 Hz, 1H), 7.70 (br s, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ = 13.8, 22.3, 26.9, 33.7, 70.3, 179.3 ppm; $C_6H_{12}O_3$: 132.15.

2-hydroxydecanoic acid: Yld: 81%; mp: 76 °C; IR (KBr): $\tilde{\nu}$ = 3400 (OH), 2942 ($C_{al}H$), 2848 ($C_{al}H$), 1721 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0.86 (t, ^3J = 6.1 Hz, 3H), 1.25 (m, 12H), 1.76 (m, 2H), 4.25 (dd, ^3J = 6.3 Hz, ^3J = 4.5 Hz, 1H), 7.14 (s broad, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ = 14.1, 22.7, 24.8, 29.3, 29.4, 31.0, 31.9, 34.2, 76.3, 179.3 ppm; $C_{10}H_{20}O_3$: 188.26.

2(*R*)-hydroxyacids: Enantiopure 2(*R*)-hydroxyhexanoic acid was obtained by enzymatic resolution by using the *Candida Cylindracea* lipase in toluene, according to the procedure of Dordick and Parida.[29] 2(*R*)-hydroxydecanoic acid was obtained by chemical resolution by using 1(*R*)-methylbenzylamine, according to the procedure of Kellyand and Lacour.[30] Subsequent acetylation afforded the corresponding 2(*R*)-acetoxyacids **10a**, **b**.

Typical procedure for 2(*R*)-acetoxyacids ((*R*)-**10a**, **b**): A solution of (*R*)-hydroxyacid (2.8 mmol), acetic anhydride (5.6 mmol), and dimethylaminopyridine (0.28 mmol) in pyridine (10 mL) was stirred for 24 h at 25 °C then diluted with CH_2Cl_2 (50 mL) and successively washed with aqueous 5 N HCl, saturated NaCl (50 mL) and a saturated aqueous solution of $CuSO_4$ (50 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was distilled under reduced pressure to afford 2(*R*)-acetoxyacid (*R*)-**10** as a clear oil.

2(*R*)-acetoxyhexanoic acid ((*R*)-**10a**): Yld: 79%; $bp_{0.2}$: 81 °C; $[\alpha]_D^{20}$ = + 14.8 (*c* = 1.3, $CHCl_3$); IR (film): $\tilde{\nu}$ = 3400 (OH), 2960 ($C_{al}H$), 2870 ($C_{al}H$), 1740 (C=O), 1712 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0.92 (t, ^3J = 6.8 Hz, 3H), 1.27 (m, 4H), 1.55 (dd, ^3J = 14.1, 6.6 Hz, 2H), 2.14 (br s, 1H), 4.36 (t, ^3J = 6.6 Hz, 1H), 9.80 (br s, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ = 13.8, 20.3, 20.5, 27.1, 30.6, 71.8, 170.7, 174.0; $C_8H_{14}O_4$: 174.19.

2(*R*)-acetoxydecanoic acid ((*R*)-**10b**): Yld: 71%; $bp_{0.01}$: 105 °C; $[\alpha]_D^{20}$ = + 16.9 (*c* = 1.8, $CHCl_3$); IR (film): $\tilde{\nu}$ = 3400 (OH), 2958 ($C_{al}H$), 2934 ($C_{al}H$), 2850 ($C_{al}H$), 1738 (C=O), 1719 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0.81 (t, ^3J = 6.6 Hz, 3H), 1.31 (m, 12H), 1.77 (m, 2H), 1.97 (s, 3H), 4.93 (t, ^3J = 6.6 Hz, 1H), 10.27 (br s, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ = 14.0, 20.5, 22.6, 25.1, 29.1, 29.2, 29.3, 30.9, 31.8, 72.0, 170.4, 175.8 ppm; $C_{12}H_{22}O_4$: 230.30.

General procedure for ceramide analogues 11a-c, e-g: Under an inert atmosphere, the coupling agent (see below for details; 0.22 mmol) in CH_2Cl_2 (5 mL) was added to an ice-cooled solution of **9** (0.20 mmol), 2(*R*)-acetoxyacid (*R*)-**10** (0.22 mmol), and diisopropylethylamine (0.40 mmol) in CH_2Cl_2 (10 mL). After stirring for 24 h at 25 °C, the mixture was diluted with AcOEt (50 mL) and washed successively with HCl (1 N aq, 2 × 30 mL), saturated aq $NaHCO_3$ (2 × 30 mL), and brine (30 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. **11** was obtained by silica-gel column chromatography (petroleum ether/AcOEt, 2:1).

Sample data for 11a:

Coupling agent: HDTU; Yld: 86%; colorless solid, mp: 82 °C; R_f = 0.8 (AcOEt/petroleum ether, 1:1); $[\alpha]_D^{20}$ = + 1.8 (*c* = 0.8, $CHCl_3$); IR (KBr): $\tilde{\nu}$ = 3600 – 3300 (OH, NH), 3058 ($C_{ar}H$), 2960 ($C_{al}H$), 2860 ($C_{al}H$), 1746 (C=O), 1732 (C=O), 1648 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0,91 (m, 6H), 1.33 (m, 14H), 1.70 (m, 2H), 2.10 (s, 3H), 3.27 (br s, 1H), 4.14 (m, 3H), 4.56 (m, 1H), 4.88 (m, 1H), 6.28 (d, ^3J = 8.6 Hz, 1H), 7.42 (m, 3H), 7.72 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ = 13.9, 14.0, 20.6, 22.2, 22.5, 22.6, 27.0, 29.2, 30.4, 31.6, 34.0, 52.2, 63.7, 69.6, 72.6, 127.1, 128.4, 131.2, 134.2, 158.5, 167.9, 171.0 ppm; MS: *m/z* (%): 436 (90) $[MH]^+$, 262 (40) $[C_{16}H_{24}NO_3]^+$, 105 (100) $[PhCO]^+$, 93 (58) $[C_7H_9]^+$, 43 (39) $[C_2H_3O]^+$; $C_{24}H_{37}NO_6$: 435.56.

Analytical data for compounds **11b**, **c**, **e – g** are provided in the Supporting Information.

Analogue of ceramide 11d:

Under an inert atmosphere, TMSCl (1,1 mL, 0.37 mmol) was added to a solution of **9a** (100 mg, 0.34 mmol) and triethylamine (82 mg, 0.82 mmol) in CH_2Cl_2 (10 mL). After stirring for 2 h at 25 °C, the mixture was ice-cooled and a solution of decanoyl chloride (78 mg, 0.41 mmol) in CH_2Cl_2 (5 mL) was added. The mixture was stirred for 1 h at 0 °C and then methanol (5 mL) and TMSCl (1,1 mL, 0.37 mmol) were added. After stirring for 2 h at 25 °C, CH_2Cl_2 (20 mL) was added and the solution was washed successively with HCl (1 N, aq, 10 mL) and brine (10 mL). The organic layer was dried over $MgSO_4$ and evaporated in vacuo. After silica-gel column chromatography (petroleum ether/AcOEt, 2:1), **11d** (90 mg, 63%) was obtained as a colorless solid. R_f = 0.4 (petroleum ether/AcOEt, 2:1); mp: 76 °C; $[\alpha]_D^{20}$ = + 0.5 (*c* = 3.2, $CHCl_3$); IR (KBr): $\tilde{\nu}$ = 3400 (OH, NH), 3010 ($C_{ar}H$), 2927 ($C_{al}H$), 2849 ($C_{al}H$), 1745 (C=O), 1641 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0.83 (m, 6H), 1.10 – 1.51 (m, 24H), 2.32 (t, ^3J = 7,4 Hz, 2H), 3.76 (m, 1H), 4.35 (m, 3H), 6.12 (m, 1H), 7.45 (m, 3H), 7.75 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ = 14.0, 22.5, 25.1, 25.8, 26.0, 29.1, 29.2, 29.3, 29.5, 30.6, 31.1, 31.7, 31.9, 53.9, 63.4, 69.5, 125.2, 128.4, 131.6, 134.4, 166.8, 170.9 ppm; $C_{26}H_{43}NO_4$: 433.63.

General procedure for protected glycosphingolipid analogues II a – g:

Under an inert atmosphere, $BF_3 \cdot Et_2O$ (170 mg, 1.20 mmol) was added to an ice-cooled solution of ceramide **11** (0.20 mmol) and penta-*O*-acetyl-β-D-galactopyranose (78 mg, 0.20 mmol) in CH_2Cl_2 (30 mL). The mixture was stirred for 4 h at 25 °C and then washed with brine (20 mL), dried over $MgSO_4$, and concentrated in vacuo. After silica-gel column chromatography (petroleum ether/AcOEt, 1:1), the protected analogue of glycosphingolipid **II** was obtained.

Sample data for IIa:

Yld: 69%; brown oil; R_f = 0.5 (AcOEt/petroleum ether, 1:1); $[\alpha]_D^{20}$ = + 1.6 (*c* = 0.3, $CHCl_3$); IR (film): $\tilde{\nu}$ = 3450 (NH), 3089 ($C_{ar}H$), 2991 ($C_{al}H$), 2928 ($C_{al}H$), 2854 ($C_{al}H$), 1760 – 1740 (C=O), 1679 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 0.87 (m, 6H), 1.31 (m, 16H), 2.04 – 2.16 (m, 15H), 4.11 (m, 7H), 4.45 (d, ^3J = 7.1 Hz, 1H), 5.01 (m, 2H), 5.52 (m, 2H), 6.32 (d, ^3J = 8.1 Hz, 1H), 7.31 (m, 3H), 7.81 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ = 13.9, 14.0, 20.4, 20.5, 20.6, 22.0, 25.4, 27.1, 29.2, 30.6, 31.6, 34.3, 51.9, 61.1, 61.5, 67.0, 68.6, 69.7, 69.9, 70.4, 70.9, 101.2, 127.1, 128.4, 131.3, 136.1, 165.7, 169.9, 171.1 ppm; MS: *m/z* (%): 766 (10) $[MH]^+$, 436 (25) $[C_{24}H_{38}NO_6]^+$, 331 (60) $[C_{14}H_{19}O_9]^+$, 185 (90) $[C_9H_{15}NO_3]^+$, 93 (100) $[C_7H_9]^+$; $C_{38}H_{55}NO_{15}$: 765.85.

Analytical data for compounds **II b – g** are provided in the Supporting Information.

General procedure for unprotected glycosphingolipid analogues 12 a – g:

A solution of protected analogue **II** (0.10 mmol) and K_2CO_3 (0.70 mmol) in methanol (20 mL) was stirred under an inert atmosphere for 24 h at 25 °C. DOWEX 50W/8 (0.5 g) was added and the mixture was filtered through Celite. The filtrate was evaporated in vacuo and, after recrystallization in methanol and lyophilization, the unprotected analogue **12** was obtained as a pale yellow powder.

Analogue **12 a**: Yld: 61 %; mp: > 250 °C; IR (KBr): $\tilde{\nu} = 3400$ (OH, NH), 2912 ($C_{al}H$), 2810 ($C_{al}H$), 1658 (C=O) cm^{-1}; ^1H NMR (CD_4O/D_2O): $\delta =$ 0.88 (m, 6H), 1.22 – 1.39 (m, 16H), 3.37 – 3.51 (m, 3H), 3.89 – 4.22 (m, 9H), 7.36 (m, 1H) ppm; MS: *m/z* (%): 452 (0) $[M]^+$, 384 (100) $[C_{21}H_{37}NO_5]^+$, 317 (40) $[C_{15}H_{27}NO_6]^+$, 131 (35) $[C_6H_{12}NO_2]^+$, 39 (50) $[C_2NH]^+$; $C_{21}H_{41}NO_9$: 451.55.

Analogue **12 b**: Yld: 60 %; mp: > 250 °C. IR (KBr): $\tilde{\nu} = 3400$ (OH, NH), 2963 ($C_{al}H$), 2856 ($C_{al}H$), 1667 (C=O) cm^{-1}; ^1H NMR (CD_4O/C_2D_6SO): $\delta = 0.99$ (m, 6H), 1.29 – 1.50 (m, 24H), 3.50 – 3.75 (m, 3H), 3.89 – 4.20 (m, 9H), 7.24 (m, 1H) ppm; MS: *m/z* (%): 508 (5) $[M]^+$, 356 (20) $[C_{20}H_{38}NO_4]^+$, 318 (60) $[C_{15}H_{27}O_7]^+$, 249 (70) $[C_9H_{15}NO_7]^+$, 39 (100) $[C_2NH]^+$; $C_{25}H_{49}NO_9$: 507.66.

Analogue **12 c**: Yld: 57 %; mp: 230 °C; IR (KBr): $\tilde{\nu} = 3370$ (OH, NH), 2916 ($C_{al}H$), 2819 ($C_{al}H$), 1661 (C=O) cm^{-1}; ^1H NMR (CD_4O/D_2O): $\delta =$ 0.78 (m, 6H), 1.21 – 1.43 (m, 18H), 3.35 – 3.52 (m, 3H), 3.87 – 4.16 (m, 8H), 7.39 (m, 1H) ppm; MS: *m/z* (%): 436 (0) $[M]^+$, 223 (80) $[C_8H_{16}NO_6]^+$, 185 (100) $[C_{10}H_{19}NO_2]^+$, 131 (100) $[C_5H_{10}NO_3]^+$, 93 (100) $[C_3H_9O_3]^+$, 39 (65) $[C_2NH]^+$; $C_{21}H_{41}NO_8$: 435.55.

Analogue **12 d**: Yld: 45 %; mp: > 250 °C; IR (KBr): $\tilde{\nu} = 3400$ (OH, NH), 2981 ($C_{al}H$), 2863 ($C_{al}H$), 1672 (C=O) cm^{-1}; ^1H NMR ($CD_4O/D_2O/C_2D_6SO$): $\delta = 0.83$ (m, 6H), 1.20 – 1.48 (m, 26H), 3.42 – 3.55 (m, 3H), 3.96 – 4.36 (m, 8H), 7.35 (m, 1H); MS: *m/z* (%): 492 (25) $[M]^+$, 386 (100) $[C_{22}H_{43}NO_4]^+$, 262 (28) $[C_{10}H_{16}NO_7]^+$; $C_{25}H_{49}NO_8$: 491.66.

Analogue **12 e**: Yld: 57 %; mp: 210 °C; IR (KBr): $\tilde{\nu} = 3350$ (OH, NH), 2936 ($C_{al}H$), 2841 ($C_{al}H$), 1664 (C=O) cm^{-1}; ^1H NMR ($CD_4O/D_2O/CDCl_3$): $\delta = 0.93$ (m, 6H), 1.29 (m, 16H), 3.48 – 3.55 (m, 3H), 3.90 – 4.18 (m, 9H), 7.18 (m, 1H); MS: *m/z* (%): 452 (0) $[M]^+$, 384 (100) $[C_{21}H_{37}NO_5]^+$, 317 (32) $[C_{15}H_{27}NO_6]^+$, 232 (25) $[C_{10}H_{18}NO_5]^+$, 131 (30) $[C_6H_{12}NO_2]^+$, 39 (45) $[C_2NH]^+$; $C_{21}H_{41}NO_9$: 451.55.

Analogue **12 f**: Yld: 48 %; mp: > 250 °C; IR (KBr): $\tilde{\nu} = 3400$ (OH, NH), 2967 ($C_{al}H$), 2872 ($C_{al}H$), 1684 (C=O) cm^{-1}; ^1H NMR (CD_4O/C_2D_6SO): $\delta = 0.90$ (m, 6H), 1.20 – 1.45 (m, 24H), 3.40 – 3.60 (m, 3H), 3.90 – 4.30 (m, 9H), 7.19 (m, 1H) ppm; MS: *m/z* (%): 508 (2) $[M]^+$, 356 (25) $[C_{20}H_{38}NO_4]^+$, 318 (35) $[C_{15}H_{27}O_7]^+$, 265 (55) $[C_{10}H_{19}NO_7]^+$, 249 (30) $[C_9H_{15}NO_7]^+$, 39 (100) $[C_2NH]^+$; $C_{25}H_{49}NO_9$: 507.66.

Analogue **12 g**: Yld: 73 %; mp: 210 °C; IR (KBr): $\tilde{\nu} = 3400$ (OH, NH), 2912 ($C_{al}H$), 2810 ($C_{al}H$), 1658 (C=O) cm^{-1}; ^1H NMR (CD_4O/D_2O): $\delta =$ 0.97 (m, 6H), 1.28 – 1.50 (m, 18H), 3.48 – 3.61 (m, 3H), 3.92 – 4.31 (m, 8H), 7.48 (m, 1H) ppm; MS: *m/z* (%): 436 (0) $[M]^+$, 223 (25) $[C_8H_{16}NO_6]^+$, 131 (100) $[C_5H_{10}NO_3]^+$, 93 (95) $[C_3H_9O_3]^+$, 39 (55) $[C_2NH]^+$; $C_{21}H_{41}NO_8$: 435.55.

Surface pressure measurements:

The surface pressure was measured with a Langmuir film balance (A&D Instruments, Oxford, UK) by using the Collect software (Labotronics Inc., Guelph, Ontario, Canada). GalCer was dissolved in a mixture of hexane/chloroform/ethanol (11:5:4) then spread inside a Teflon tank. In all experiments, the subphases were pure water obtained by filtration through a milli-Q water purification system (Millipore, Saint-Quentin, France).

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