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Dual-Action Lipophilic Iminosugar Improves Glycemic Control in Obese Rodents by Reduction of Visceral Glycosphingolipids and Buffering of Carbohydrate Assimilation

Tom Wennekes,[†] Alfred J. Meijer,[‡] Albert K. Groen,[‡] Rolf G. Boot,[‡] Johanna E. Groener,[‡] Marco van Eijk,[‡] Roelof Ottenhoff,[‡] Nora Bijl,[‡] Karen Ghauharali,[‡] Hang Song,[†] Tom J. O'Shea,[§] Hanlan Liu,[§] Nelson Yew,[§] Diane Copeland,[§] Richard J. van den Berg,[†] Gijsbert A. van der Marel,[†] Herman S. Overkleeft,[†] and Johannes M. Aerts*[,‡]

[†]*Gorlaeus Laboratories, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands,* [‡]*Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands, and* [§]*Drug and Biomaterial R&D, Genzyme, Waltham, Massachusetts*

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The lipophilic iminosugar *N*-[5-(adamantan-1-ylmethoxy)pentyl]-1-deoxynojirimycin (**2**, AMP-DNM) potently controls hyperglycemia in obese rodent models of insulin resistance. The reduction of visceral glycosphingolipids by **2** is thought to underlie its beneficial action. It cannot, however, be excluded that concomitant inhibition of intestinal glycosidases and associated buffering of carbohydrate assimilation add to this. To firmly establish the mode of action of **2**, we developed a panel of lipophilic iminosugars varying in configuration at C-4/C-5 and N-substitution of the iminosugar. From these we identified the L-*ido* derivative of **2**, L-*ido*-AMP-DNM (**4**), as a selective inhibitor of glycosphingolipid synthesis. Compound **4** lowered visceral glycosphingolipids in ob/ob mice and ZDF rats on a par with **2**. In contrast to **2**, **4** did not inhibit sucrase activity or sucrose assimilation. Treatment with **4** was significantly less effective in reducing blood glucose and HbA1c. We conclude that the combination of reduction of glycosphingolipids in tissue and buffering of carbohydrate assimilation by **2** produces a superior glucose homeostasis.

Introduction

Coinciding with obesity, type 2 diabetes has reached epidemic proportions worldwide. Insulin resistance is one of the earliest detectable abnormalities during the development of type 2 diabetes. The precise cause for the rapidly increasing occurrence of insulin resistance has not been firmly established, but there is growing evidence that obesity and associated lipotoxicity play a crucial role.[1] Recent literature links insulin resistance in tissues to the presence of excessive amounts of a particular group of lipids, called glycosphingolipids. These lipids are enriched in detergent resistant membranes, in physical proximity to the insulin receptor.[2] A regulatory role for glycosphingolipids, in particular the ganglioside GM3, in insulin sensitivity is substantiated by a rapidly growing body of experimental evidence.[3] Interaction of gangliosides and the insulin receptor was originally described by Nojiri et al., demonstrating the ganglioside-mediated inhibition of insulin-dependent cell growth of leukemic cell lines.[4] Tagami and co-workers were the first to demonstrate that addition of GM3 to cultured adipocytes suppresses phosphorylation of the insulin receptor and its downstream substrate insulin receptor substrate-1 (IRS-1[a]), resulting in reduced glucose uptake.[5] Inokuchi and co-workers reported that exposure of cultured adipocytes to tumor necrosis factor-α (TNF-α) increases GM3 and inhibits insulin receptor (IR) and IRS-1 phosphorylation. This was found to be counteracted by 1-phenyl-2-decanoylamino-3-morpholinopropanol (PDMP), an inhibitor of glycosphingolipid biosynthesis.[6] Mutant mice lacking GM3 have been reported to show an enhanced phosphorylation of the skeletal muscle insulin receptor after ligand binding and to be protected from high-fat-diet-induced insulin resistance.[7] Consistent with this is the recent report on improved insulin sensitivity and glucose tolerance in mice with increased expression of the GM3 degrading sialidase Neu3.[8] Conversely, GM3 levels are elevated in the muscle of certain obese, insulin resistant mouse and rat models.[3] Altered sphingolipid metabolism, reflected by increased glycosphingolipid levels, has recently also been documented in relation to neuronal pathology in diabetic retinopathy.[9] Very recently Kabayami et al. provided evidence that the interaction of GM3 with the insulin receptor is mediated by a specific lysine residue located just above the transmembrane domain of the receptor and that excess levels of GM3 promote dissociation of the insulin receptor from caveolae, a location that is essential for insulin signal transduction.[10]

The value of pharmacological lowering of excessive glycosphingolipid levels to improve insulin sensitivity has recently been demonstrated by others and us.[3,11−13] Holland and co-workers reported that inhibition of the synthesis of ceramide, the precursor of glycosphingolipids, markedly improves glucose tolerance and prevents the onset of overt diabetes in obese rodents.[12] Zhao et al. demonstrated that inhibition of the first step in the biosynthesis of glycosphingolipids,

*To whom correspondence should be addressed. Phone: +31205665156. Fax: +31206915519. E-mail: j.m.aerts@amc.uva.nl.

[a] Abbreviations: AMP, 5-(adamantan-1-ylmethoxy)pentyl; ob/ob mice, obese leptin deficient mice; ZDF, Zucker diabetic fatty; HbA1c, glycated hemoglobin; IRS-1, insulin receptor substrate-1, TNF-α, tumor necrosis factor-α; IR, insulin receptor; GCS, glucosylceramide synthase; GBA1, glucocerebrosidase; GBA2, β-glucosidase 2 or non-lysosomal glucocerebrosidase; HOMA-IR, homeostatic model assessment for insulin resistance; GSLs, glycosphingolipids; AUC, area under the curve; OGT, oral glucose tolerance.

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catalyzed by glucosylceramide synthase (GCS), exerts beneficial effects. The GCS inhibitor Genz-123346 (L-tartaric acid salt of **1**, Figure 1) lowered blood glucose and glycated hemoglobin (HbA1c) levels and improved glucose tolerance in insulin resistant rodents.[13] Finally, we showed that treatment of various rodent models of insulin resistance with the lipophilic iminosugar AMP-DNM (**2**, Figure 1), a well tolerated and potent inhibitor of GCS, very markedly lowered circulating glucose levels, improved oral glucose tolerance (OGT), reduced HbA1c, and improved insulin sensitivity in muscle and liver.[11] In addition, treatment with **2** was found to cause a marked improvement in insulin sensitivity of adipocytes and to reduce inflammation in adipose tissue of obese mice.[14]

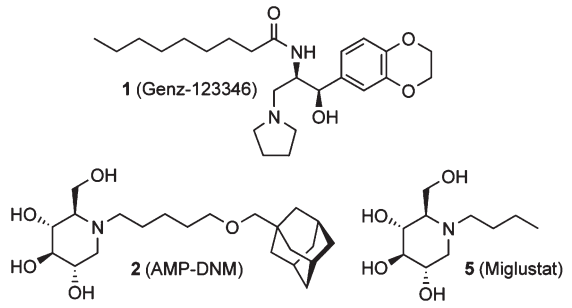


Figure 1. Structures of **1**, **2**, and **5**.

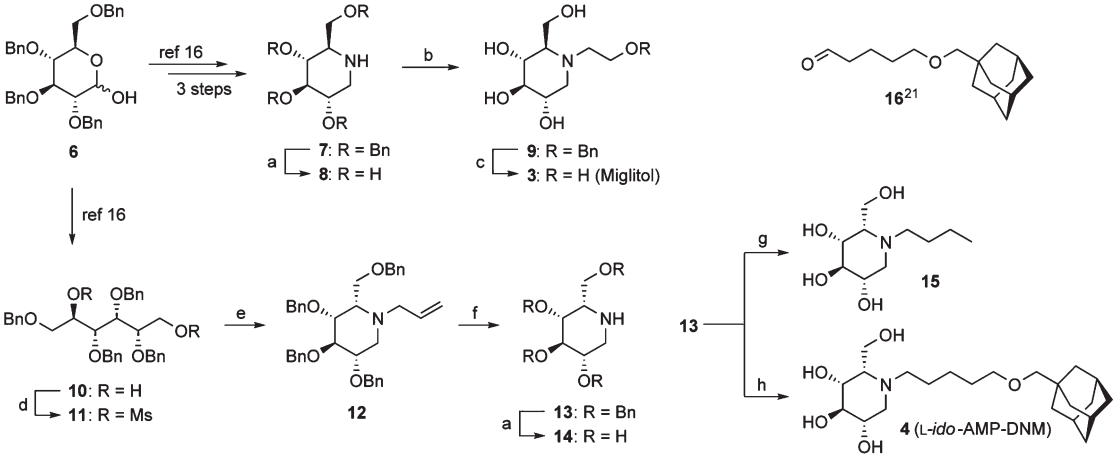
The marked beneficial effect of **2** on glycemic control in obese mice might not only be exerted by reduction of glycosphingolipids in tissues. The same compound was found to inhibit in vitro the enzymatic activities of some glycosidases like sucrase and maltase.[15,16] The latter effect is similar to the mode of action of registered antidiabetics, including the iminosugar miglitol (**3**, Scheme 1).[17] To establish whether concomitant reduction of carbohydrate assimilation by **2** contributes to its beneficial effect on glycemic control, we looked for an analogue of **2** that inhibited GCS more specifically. The archetypal iminosugar, 1-deoxynojirimycin (**8**, Scheme 1) as well as the piperidine ring **2**, **3**, and the registered GCS inhibitor miglustat (**5**, Figure 1)[18] all possess D-gluco stereochemistry. It is a well established fact that structural mimicry of the substrate is one of the causes for inhibition of intestinal glycosidases by this type of iminosugar. Therefore, changing the iminosugar stereochemistry could be a means to develop more specific GCS inhibitors. Platt, Butters, and coworkers have demonstrated previously that *N*-butyl-D-*galacto*-1-deoxynojirimycin (**21**, Scheme 2), a C-4 epimer of **5**, still inhibits GCS.[19] The same has also been reported for *N*-pentyl-L-*ido*-1-deoxynojirimycin, a *N*-pentyl substituted C-5 epimer of **5**.[20] Therefore, we chose to develop a panel of nine structural and stereochemical analogues of lead compound **2**. The panel consisted of all three C-4/C-5 epimers of 1-deoxynojirimycin (D-*galacto*, L-*altro*, and L-*ido*) that either had their endocylic nitrogen unsubstituted or substituted with a butyl for analogues of **5** or a 5-(adamantan-1-ylmethoxy)pentyl (AMP) group for analogues of **2**.

We here demonstrate that both the D-*galacto* (C-4 epimer) and L-*ido* (C-5 epimer) analogues of **2** are still potent inhibitors of GCS. Of these, L-*ido*-AMP-DNM (**4**, Scheme 1) no longer inhibits the intestinal glycosidases, making it specific for GCS. Consequently, **4** was compared head-to-head with **2** in ob/ob mouse and ZDF rat models of insulin resistance and type 2 diabetes. The ability of these compounds to improve insulin sensitivity and buffer sucrose assimilation was examined. Their effects on glycemic control were also compared with those of **1** and two registered deoxynojirimycin-type drugs **5** and **3**. The results of these investigations indicate that the prominent beneficial effect of **2** on glycemic control results from dual inhibition of both carbohydrate assimilation and visceral glycosphingolipid biosynthesis.

Results

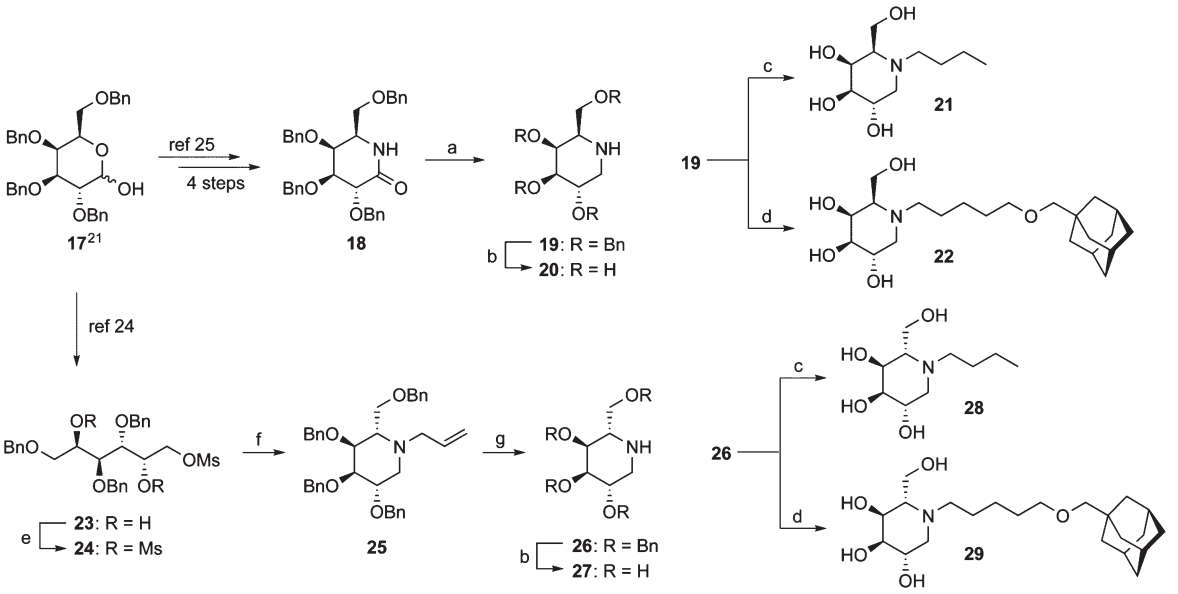
Synthesis of the Iminosugars. Lead compound **2** was synthesized starting from commercially available 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (**6**) according to our previously reported route.[21] 1-Deoxynojirimycin (**8**) was obtained by Pd/C catalyzed hydrogenolysis of known 2,3,4,6-tetra-*O*-benzyl-1-deoxynojirimycin (**7**) (Scheme 1).[16] The synthesis of **3** started with a $NaCNBH_3$ mediated reductive amination of **8** with commercially available 2-(benzyloxy)acetaldehyde to provide **9**. Hydrogenolysis of **9** provided **3**. Compound **5** was commercially available and used as received.

Scheme 1. Synthesis of Iminosugars with 1-Deoxynojirimycin (D-*gluco*) **3**, **8** and L-*ido* **4**, **14**, **15** Stereochemistry[a]



[a] Reagents and conditions: (a) Pd/C, 4 bar of H_2, EtOH, aq HCl, 20 h. **8**: 93%. **14**: 86%. (b) 2-(Benzyloxy)acetaldehyde, $NaCNBH_3$, MeOH/AcOH, 48 h, 86%; (c) Pd/C, 4 bar of H_2, MeOH/H_2O, aq HCl, 20 h, 95%; (d) MsCl, pyridine, 0 °C, 2 h; (e) allylamine, reflux, 20 h, 67% over two steps; (f) (1) KO^{t}Bu, DMSO, 100 °C, 30 min; (2) 1 M aq HCl, 15 min, 81%; (g) (1) butyraldehyde, $NaCNBH_3$, CH_3CN/AcOH, 20 h; (2) BCl_3, CH_2Cl_2, 0 °C, 20 h, 82% over two steps; (h) (1) **16**, Pd/C, 4 bar of H_2, EtOH/AcOH, 20 h; (2) Pd/C, 4 bar of H_2, EtOH, aq HCl, 20 h, 77% over two steps.

Scheme 2. Synthesis of Iminosugars with D-*galacto* **20**−**22** and L-*altro* **27**−**29** Stereochemistry[a]



[a] Reagents and conditions: (a) $LiAlH_4$, THF, reflux, 3 h, 71%; (b) BCl_3, CH_2Cl_2, 0 °C, 20 h. **20**: 96%. **27**: 77%. (c) (1) Butyraldehyde, $NaCNBH_3$, CH_3CN/AcOH, 20 h; (2) BCl_3, CH_2Cl_2, 0 °C, 20 h. **21**: 74%. **28**: 91% over two steps. (d) (1) **16**, $NaCNBH_3$, CH_3CN/AcOH, 20 h; (2) Pd/C, 4 bar of H_2, EtOH, aq HCl, 20 h. **22**: 61%. **29**: 89% over two steps. (e) MsCl, pyridine, 0 °C, 2 h; (f) allylamine, reflux, 20 h, 82% over two steps; (g) (1) KO^{t}Bu, DMSO, 100 °C, 30 min; (2) 1 M aq HCl, 15 min, 73%.

Table 1. Apparent IC_{50} Values in μM for 1-Deoxynojirimycin (D-*gluco*)-, L-*ido*-, D-*galacto*-, and L-*altro*-Based Iminosugars[a]

Iminosugar	GCS	Sucrase	Maltase	Lactase	GBA1	GBA2	Lysosomal acid α-glucosidase	De-branching enzyme
Compound number and structure	*in vivo*	*in vitro*	*in vitro*	*in vitro*	*in vitro*	*in vitro*	*in vitro*	*in vitro*
8: R = H	>100	2	2	62	250	21	1.5	10
3: R = Ethanol	>1000	0.5	6	50	200	-	2.0	-
5: R = Butyl	50	0.5	9	> 100	400	0.230	0.1	10
2: R = AMP[a]	0.15	0.5	4	35	0.2	0.001	0.4	10
14: R = H	9	1000	> 1000	> 1000	> 1000	400	> 1000	> 100
15: R = Butyl	12	1000	> 1000	1000	> 1000	0.25	> 1000	> 100
4: R = AMP[a]	0.15	> 1000	1000	> 1000	2	< 0.001	> 1000	> 100
20: R = H	>100	0.26	1.5	50	350	100	6	10
21: R = Butyl	10	0.46	10	1000	320	0.3	1.5	10
22: R = AMP[a]	0.5	3.5	15	375	0.2	< 0.001	0.5	4
27: R = H	>100	220	> 1000	400	> 1000	100	> 1000	> 100
28: R = Butyl	>100	450	> 1000	> 1000	> 1000	9	500	> 100
29: R = AMP[a]	>100	1000	> 1000	> 1000	30	0.5	500	> 100

[a] AMP = 5-(adamantan-1-ylmethoxy)pentyl.

The three L-*ido*-1-deoxynojirimycin iminosugars (**4**, **14**, and **15**) were synthesized starting from 2,3,4,6-tetra-*O*-benzyl-D-glucitol **10**, which in turn could be obtained by reduction of **6** (Scheme 1). Transformation of the two hydroxyl functions in **10** into their methanesulfonic esters and refluxing the obtained crude dimesylate **11** in allylamine provided **12**.[22,23] Isomerization and cleavage of the allyl-function in **12** using in situ generated dimsyl anions followed by acidic workup provided **13**. Hydrogenolysis of **13** with catalytic Pd/C provided **14**. Reductive amination of **13** with butyraldehyde under the agency of $NaCNBH_3$ and subsequent deprotection of the crude intermediate with BCl_3 provided **15**. Synthesis of

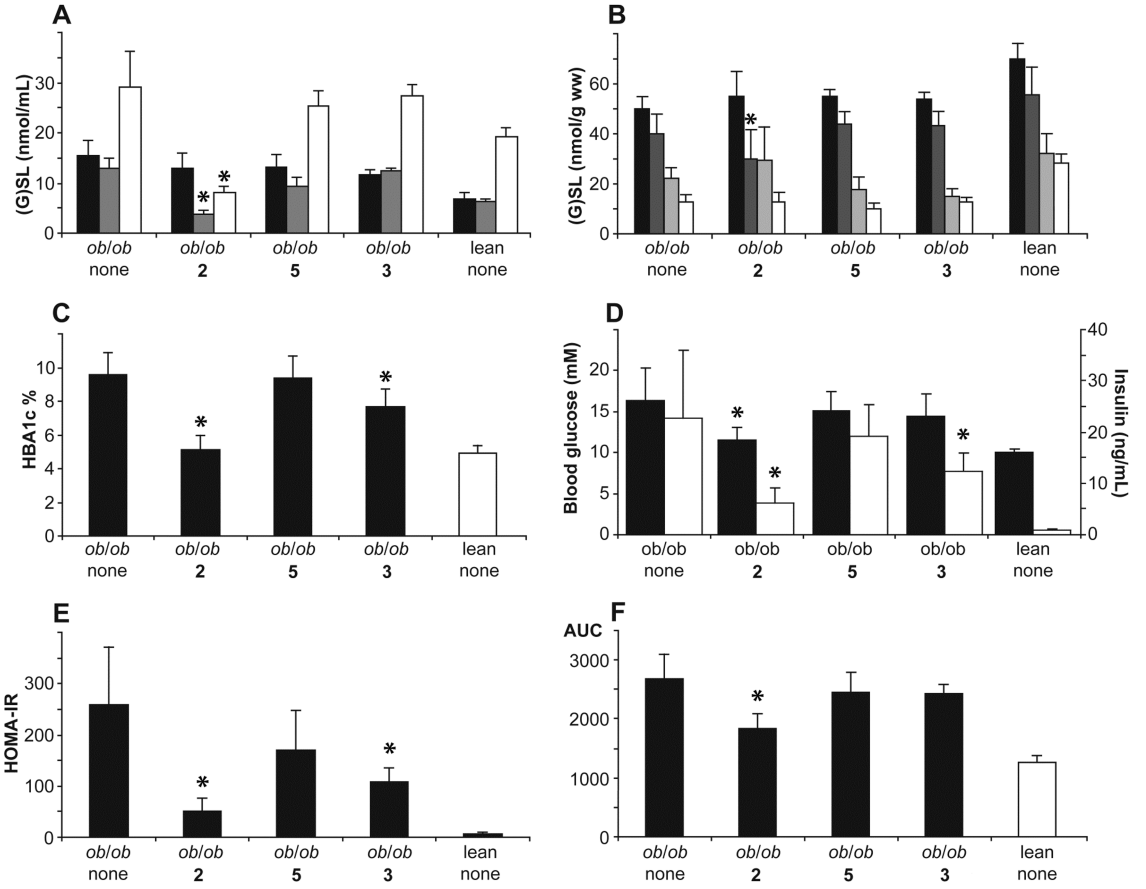


Figure 2. Effects of **2**, **5**, and **3** treatment on (G)SLs and glycemic control in ob/ob mice and comparative values in untreated normal mice. Animals were treated for 4 weeks daily with 100 mg of compound per kg body weight. (A) Plasma content (nmol/mL) of ceramide and GSLs: ceramide (black); glucosylceramide (gray); total gangliosides (white). (B) Liver content (nmol/g) of ceramide and GSLs (left to right): ceramide/10; glucosylceramide; GM2; GM2-glycol/10. (C) HbA1c. (D) Blood glucose (black) and insulin (white). (E) HOMA-IR index. (f) Oral glucose tolerance (OGT, area under the curve). Asterisk (*) indicates significant ($p < 0.05$) change by treatment compared to untreated ob/ob mice.

4 was achieved by a reductive amination between **13** and 5-(adamantane-1-ylmethoxy)-1-pentanal (**16**)[21] by selective Pd/C catalyzed hydrogenolysis of the intermediate imine in the presence of acetic acid. Deprotection of the benzyl ethers in the crude intermediate by Pd/C catalyzed hydrogenolysis in the presence of hydrochloric acid produced **4**.

The synthesis of the three D-*galacto*-iminosugars (**20**, **21**, and **22**) commenced with the preparation and subsequent transformation of 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose **17**[24] into **18**[25] using literature procedures (Scheme 2). Lactam **18** was reduced to **19** with $LiAlH_4$ in refluxing THF. Deprotection of the benzyl ethers of **19** with BCl_3 provided **20**. Reductive amination of **19** with either butyraldehyde or 5-(adamantane-1-ylmethoxy)-1-pentanal (**16**) under the agency of $NaCNBH_3$ and subsequent deprotection of the crude N-alkylated intermediates produced **21** and **22**. The three L-*altro*-iminosugars (**27**, **28**, and **29**) were synthesized starting from 2,3,4,6-tetra-*O*-benzyl-D-galactitol **23**[24] using the same route as described for the L-*ido*-iminosugars (see Scheme 2).

Effects of the 1-Deoxynojirimycin-Based Iminosugars on Glycemic Control in ob/ob Mice. In the first experiment, the three existing 1-deoxynojirimycin-based iminosugars, lead compound **2**, **5**, **3**, and 1-deoxynojirimycin (**8**) itself were comparatively investigated with respect to their ability to inhibit relevant glycosidases and GCS (Table 1). As expected, the antidiabetic **3** is a potent inhibitor of the intestinal glycosidases maltase and sucrase. Compounds **8**, **5**, and **2** also inhibit these enzymes at micromolar concentrations. Lead compound **2** in particular is a potent inhibitor of GCS (IC_{50} = 150 nM), **5** a weaker inhibitor (IC_{50} = 50 000 nM), while **8** and **3** do not inhibit GCS at all.

The effects of **3**, **5**, and **2** on glucose homeostasis in obese, insulin resistant ob/ob mice were studied next. For this purpose, 7-week old C57Bl/6J (control group) and ob/ob mice were treated for 4 weeks with 100 (mg/kg)/d of **3**, **5**, or **2**. Only in the case of **2** was a significant ($p < 0.05$) lowering of plasma glycosphingolipids observed, without concomitant changes in ceramide content (Figure 2A). Mice treated with **2** showed a significantly lowered circulating blood glucose and insulin, improved oral glucose tolerance and HOMA-IR (homeostatic model assessment for insulin resistance), and reduced HbA1c (Figure 2C−F). Treatment with **5** had no significant positive effects on these parameters. Treatment with **3** resulted only in a significant, but minor, reduction of insulin, HOMA-IR, and HbA1c.

L-*ido* Analogue 4, a Potent and More Specific Inhibitor of GCS. The C-4/C-5 analogues of lead compound **2** were assayed on their inhibitory capacity toward the relevant glycosidases and GCS (Table 1). Of the nine compounds only the three D-*galacto*-iminosugars (C-4 epimer; compounds **20**−**22**) still substantially inhibit intestinal glycosidases. Of note, the observed IC_{50} value of **21** for the murine lactase is surprisingly high. In an earlier investigation,

Andersson and co-workers reported significant inhibition of porcine lactase by **21**.[26] Presently, we have no explanation for this discrepancy except for the different origin of lactase preparations used in the two studies.

All three L-*altro*-iminosugars (C-4 and C-5 epimer, compounds **27**−**29**) show very weak to no inhibition of GCS. In line with literature reports, the D-*galacto*- and L-*ido*-iminosugars (C-5 epimer, compounds **4**, **14**−**15**) do inhibit GCS. The unsubstituted D-*galacto*- and L-*ido*-iminosugars (compounds **20** and **14**) show the lowest to no inhibition of GCS. The D-*galacto*- and L-*ido*-*N*-butyl analogues (compounds **21** and **15**) are inhibitors of GCS in the micromolar range. Analogous to **5** versus **2**, a great increase in inhibitory potency for GCS is observed in switching from *N*-butyl substitution to *N*-AMP substitution of the D-*galacto*- and L-*ido*-iminosugars. Compared to **2**, the AMP-substituted D-*galacto*-iminosugar (**22**) is an only slightly less potent inhibitor of GCS, but as mentioned above, it still inhibits the intestinal glycosidases. However, of particular interest was L-*ido* analogue **4**, which did exhibit the required profile for a potent GCS-selective inhibitor. Compound **4** is slightly better than lead compound **2** with regard to inhibition of GCS ($IC_{50} < 150$ nM) but sharply contrasts from this compound in its much reduced capacity to inhibit intestinal glycosidases. Exposure of various types of cultured cells to **2** and **4** resulted in comparable lowering of glycosphingolipids without concomitant increases in ceramide (not shown). The pharmacokinetic properties of **2** and **4** were also found to be very similar (Table 2; for more details, see Supporting Information).

Table 2. Pharmokinetics of **2** and **4** in ZDF Rats

pharmokinetics	**2**	**4**	units
C_{max}	1200 ± 400	1600 ± 300	ng·mL
C_{max}/dose	120 ± 40	160 ± 30	ng·mL/(mg/kg)
$t_{1/2}$	4.2 ± 1.8	2.9 ± 2.0	h
AUC_{inf}	3400 ± 500	3300 ± 500	ng·h/mL
AUC_{inf}/dose	340 ± 50	330 ± 50	[ng·h/mL]/(mg/kg)
F (free fraction)	41 ± 7	52 ± 7	%

Comparison of Effects of Lead Compound 2 and L-*ido* Analogue 4 in ob/ob Mice and ZDF Rats. The effect of **2** and **4** on ob/ob mice was comparatively investigated. For this purpose, 7-week-old animals were treated for 4 weeks with 100 (mg/kg)/d compound. Treatment with **2** and **4** resulted in significant reductions in glycosphingolipids in plasma and liver without affecting ceramide levels (Figure 3A,B). Although clear improvements in blood glucose concentration, insulin levels, HOMA-IR, and HbA1c were observed in animals treated with **4**, these were significantly smaller than those detected in animals treated with **2** (Figure 3C−E). Oral glucose tolerance (Figure 3F) and insulin signaling in the liver (Figure 4) were comparably improved in animals treated with either **4** or **2**.

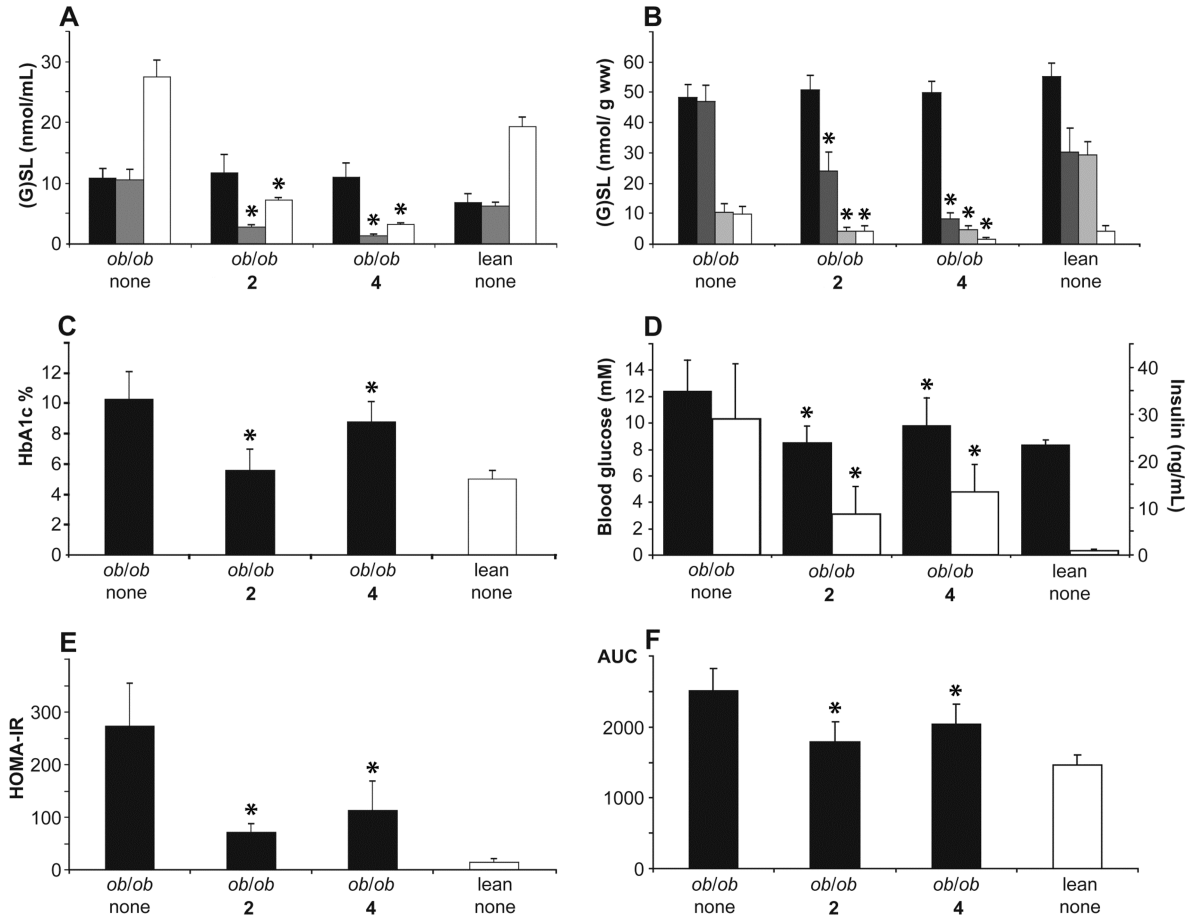


Figure 3. Effects of **2** and **4** treatment on (G)SLs and glycemic control in ob/ob mice and comparative values in untreated normal mice. Animals were treated for 4 weeks daily with 100 mg compound per kg bodyweight. (A) Plasma content (nmol/mL) of ceramide and GSLs: ceramide (black); glucosylceramide (gray); total gangliosides (white). (B) Liver content (nmol/g) of ceramide and GSLs (left to right): ceramide/10; glucosylceramide; GM2; GM2-glycol/10. (C) HbA1c. (D) Blood glucose (black) and insulin (white). (E) HOMA-IR index. (f) Oral glucose tolerance (OGT, area under the curve). Asterisk (∗) indicates significant ($p < 0.05$) change by treatment compared to untreated ob/ob mice.

Next, ZDF rats were treated with lead compound **2** and L-*ido* analogue **4**. In addition, the effect of **1**,[13,27,28] a ceramide analogue that specifically inhibits GCS and not intestinal glycosidases, was studied. For all three compounds a 4 week treatment resulted in significant reductions of plasma and liver glycosphingolipids without changes in ceramide content (Figure 5A,B). Similar to the findings with ob/ob mice, treatment of ZDF rats with **2** resulted in more prominent improvements in blood glucose concentration and HbA1c compared to treatment with **4** or **1** (Figure 5C,D).

Different Effects of Lead Compound 2 and L-*ido* Analogue 4 on Sucrose Assimilation. Next, we investigated the effect of **2** and **4** on the uptake of glucose from orally administered sucrose. For this purpose, 7-week old ob/ob mice were either treated with 100 (mg/kg)/d of compound **2** or **3** or left untreated. Sucrose (1 g/kg) was orally administered, and blood glucose was monitored. The increase of blood glucose and its clearance from the circulation were slower in mice treated with **2** than in animals treated with **4** (Figure 6A). The area under the curve (AUC) for the first hour after sucrose administration of **2** and **4** treated mice differed significantly (840 ± 65, 975 ± 75 mM·min, $p < 0.05$). After 1 week, the same animals orally received glucose (0.5 g/kg).

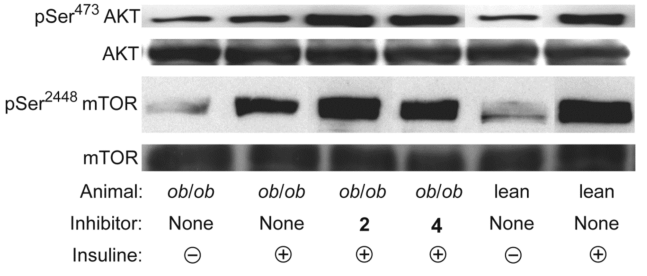


Figure 4. Effects of **2** and **4** on phosphorylation of liver Ser^{473} AKT and Ser^{2448} mTOR following insulin stimulus.

No differences were noted in the kinetics of glucose appearance and disappearance from the blood when mice treated with **2**

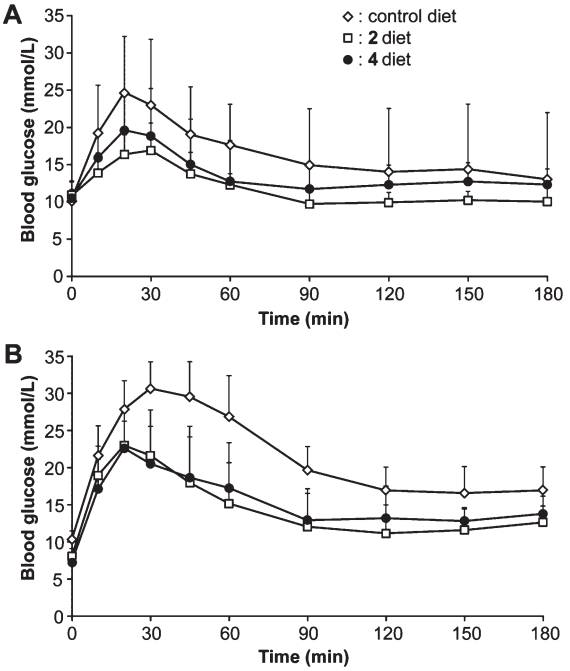


Figure 6. Effect of **2** and **4** treatment on blood glucose following exposure to oral sucrose (A) and glucose (B) administration. Animals were treated for 6 weeks daily with no iminosugar, 100 mg of **2**, or 100 mg of **4** per kg body weight. (A) At week 4, sucrose (1 g/kg) was orally administered, and blood glucose was monitored. AUC (mM·min) during the first hour was 1325 ± 210 for control diet, 840 ± 65 for diet with **2**, and 975 ± 75 for diet with **4**. (B) At week 5, the same animals orally received glucose (0.5 g/kg), and blood glucose was monitored. AUC during the first hour was 1560 ± 245 for control diet, 1090 ± 95 for diet with **2**, and 1105 ± 85 for diet with **4**.

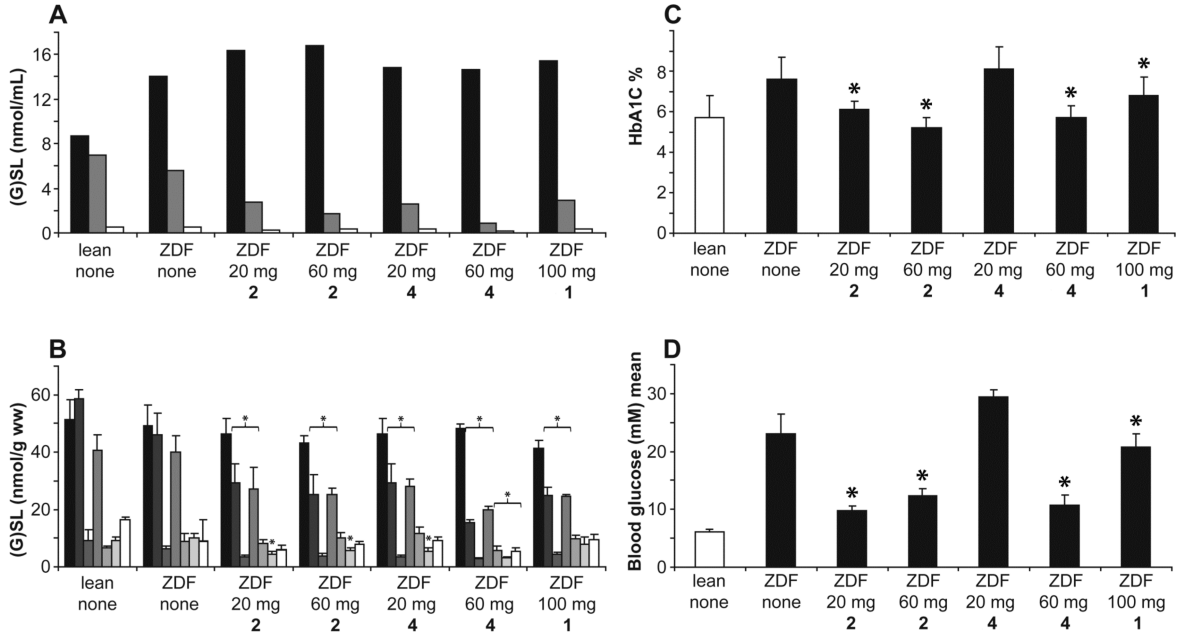


Figure 5. Effects of **2**, **4**, and **1** treatment on (G)SLs and glycemic control in ZDF rats and comparative values in untreated normal animals. Animals were treated for 4 weeks daily with indicated amount of compound per kg body weight. (A) Plasma content (pooled plasma specimens of all animals per group) of ceramide and GSLs (nmol/mL): ceramide (black); glucosylceramide (gray); total gangliosides (white). (B) Liver content (nmol/g) of ceramide and GSLs (left to right): ceramide/10; glucosylceramide; lactosylceramide; GM3; GM2; GM2-glycol/10; GD1a. (C) HbA1c. (D) Blood glucose. Asterisk (*) indicates significant ($p < 0.05$) change by treatment compared to untreated animals.

were compared with animals treated with **4** (Figure 6B). Untreated ob/ob mice showed higher blood glucose concentrations and impaired glucose tolerance upon administration of sucrose or glucose due to their insulin resistance.

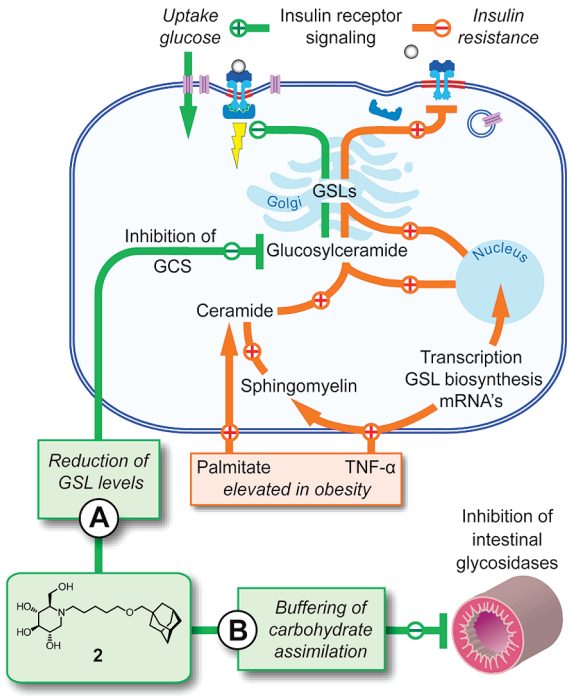
Discussion and Conclusions

In our earlier study, we have demonstrated that the GCS inhibitor, **2**, has dramatic beneficial effects on the insulin resistance and hyperglycemia seen in ZDF rats, ob/ob mice, and high-fat-diet-induced glucose-intolerant mice via a mechanism that does not require a reduction in food intake or loss of bodyweight.[11] In the ZDF type 2 diabetes model, protection of the pancreas by **2** was also observed. Given the ability of **2** to also inhibit the activity of intestinal glycosidases like sucrase in vitro, we considered the possibility that the compound might not only reduce visceral glycosphingolipids but also buffer intestinal carbohydrate assimilation in treated animals. Indeed, the assimilation of sucrose in ob/ob mice was found to be buffered in animals treated with **2**. This dual action of **2** is not surprising, since the structurally related **3** positively affects glucose homeostasis via its specific inhibition of intestinal glycosidases. On the basis of this mechanism of action, **3** is registered as antidiabetic drug.[17] Compound **3** itself does not inhibit GCS as measured with cultured cells,[11] and our present investigation rendered no indication that some metabolite is formed that causes visceral glycosphingolipid reduction. The development of **3** as antidiabetic drug was stimulated by the ancient use in the Far East of iminosugar-rich mulberry leaves to control hyperglycemia.[29] Very recently, it has indeed been demonstrated that, compared with a placebo, coingestion of mulberry extract with 75 g of sucrose reduced the increase in blood glucose observed over the initial 2 h of testing in control and type 2 diabetic subjects.[30]

The realization of dual effects exerted by **2** raised the question of the relative importance of buffering of carbohydrate assimilation on the one hand and visceral glycosphingolipid lowering on the other hand for the improved glycemic control in animals treated with **2**. To dissect the two actions of **2**, we looked into the possibility to generate more specific analogues.

Evaluation of known iminosugar-based inhibitors combined with the design and preparation of novel analogues has shown that the C-4/C-5 configuration of the iminosugar and the type of substitution on the endocyclic nitrogen are critical for potent inhibition of GCS. In general, iminosugars with D-*gluco*-, D-*galacto*, and L-*ido*-stereochemistry in combination with a hydrophobic substituent on the endocyclic nitrogen inhibited GCS. Substitution with 5-adamantane-1-ylmethoxy)pentyl (AMP) provided the most potent inhibitors of GCS. Epimerization of the C-5 position of **2** greatly reduced inhibition of intestinal glycosidases and slightly increased the inhibitory potency for GCS, making **4** the most potent iminosugar-based GCS-selective inhibitor reported to date. In order to further evaluate the effects of configurational manipulation and endocyclic nitrogen substitution on the inhibitory capacity and specificity, we tested the iminosugars reported in Table 1 on four additional enzymes known to be inhibited by **2**. These enzymes are the glycogenolysis associated debranching enzyme and lysosomal acid α-glucosidase and also the glucosylceramide catabolism related glucocerebrosidase (GBA1) and β-glucosidase 2 (GBA2).[11] The IC_{50} values of the iminosugars for these enzymes are shown in Table 1. Of note, compared to **2**, L-*ido* analogue **4** is a much poorer inhibitor of GBA1 (IC_{50} = 1.0 μM versus 0.2 μM), lysosomal acid α-glucosidase (IC_{50} > 1 mM versus 0.4 μM), and debranching enzyme (IC_{50} > 1 mM versus 10 μM), which further emphasizes its specificity in GCS inhibition.

Scheme 3. Proposed Model for Improved Glycemic Control by Dual Action (A/B) of **2**



The generation of L-*ido* analogue **4** allowed us to dissect the two actions of lead compound **2**. We established that also in obese mice **4** inhibits GCS comparably to **2** and does not effectively buffer sucrose assimilation by its lack of sucrase inhibition. Treatment of ob/ob mice and ZDF rats with **4** demonstrated that sole reduction of visceral glycosphingolipids is sufficient to induce major improvements in blood glucose, HbA1c, oral glucose tolerance, and insulin signaling in the liver. These findings are consistent with the observation that **1**, which does not affect intestinal glycosidases and carbohydrate assimilation, helps to control hyperglycemia.[13] Importantly, our study also points out that the concomitant inhibition of intestinal carbohydrate assimilation by lead compound **2** adds to its prominent beneficial effect on glycemic control (see Scheme 3).

A considerable drawback of compounds that buffer carbohydrate assimilation by virtue of inhibition of intestinal glycosidases is the associated intestinal complaints that lower drug compliance. Compound **4** does not affect intestinal glycosidases. In this respect, the compound is an appealing drug, particularly for conditions in which the exclusive lowering of visceral glycosphingolipid levels is desirable without the need for buffering of carbohydrate assimilation. Examples in this respect are the inherited glycosphingolipidoses, such as Gaucher disease, Sandhoff disease, Tay-Sachs disease, and Fabry disease.[15] In all these disorders, a particular glycosphingolipid accumulates in the lysosomes because of an inherited deficiency in a catabolic lysosomal glycosidase. Reduction of glycosphingolipid biosynthesis by inhibition of GCS is envisioned to be beneficial in all these conditions.[15,31,32] Compound **5** has been registered as orphan drug for the treatment of mild to moderate type 1 Gaucher disease and has proven to be efficacious.[18,33] Given the significantly improved features of **4** compared to **5**, such as better bioavailability, specificity, and

potency of inhibition of GCS, it seems warranted to investigate its potential as a therapeutic agent for inherited glycosphingolipidoses.

In summary, lipophilic iminosugar **2** exerts beneficial effects on glycemic control by virtue of its dual lowering of visceral glycosphingolipids and buffering of carbohydrate assimilation. This dual action is desirable for control of hyperglycemia, a hallmark of type 2 diabetes. The L-*ido* analogue of **2** we developed, **4**, specifically inhibits glycosphingolipid biosynthesis and may be of interest to intervene in inherited glycosphingolipidoses. The outcome of this study also indicates that tailored iminosugars can be developed for specific therapeutic indications by designing them to exclusively act on glycosphingolipid metabolism or on other related glycoprocessing pathways.

Experimental Section

Chemistry. The ^{1}H and ^{13}C NMR experiments were recorded on a 200/50, 300/75, 400/100, 500/125, or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard for all ^{1}H NMR measurements in $CDCl_3$ and the deuterated solvent signal for all other NMR measurements. Coupling constants (J) are given in Hz. High resolution mass spectra were recorded on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode with resolution R = 60 000 at m/z 400 (mass range m/z = 150−2000). All tested iminosugars were analyzed via a combination of HPLC and LC/MS that showed a purity of >95%. The L-tartaric acid salt of **1** was obtained from Genzyme and used as received.[13] The methanesulfonic acid salt (**2**·MSA) of lead compound **2** was synthesized as previously described and used as such in the enzyme assays and animal studies.[21] Compounds **3** and **4** were used in enzyme assays and animal studies as their hydrochloric acid salts (**3**·HCl and **4**·HCl). Compound **5** was obtained commercially and used as received (Zavesca, Actelion). The remaining iminosugars were tested in the enzyme assays as their trifluoromethanesulfonic acid salt.

***N*-Allyl-2,3,4,6-tetra-*O*-benzyl-L-*ido*-1-deoxynojirimycin (12).** Methanesulfonyl chloride (1.45 mL, 18.75 mmol) was added dropwise to a cooled (0 °C) solution of **10** (4.070 g, 7.50 mmol, prepared from **6** via a previously reported procedure)[16] in pyridine (30 mL). After TLC analysis indicated complete consumption of starting material (2 h; R_f(**10**) = 0.25 in 2:1 PE/EtOAc), water (20 mL) was added and the reaction mixture was concentrated. The residue was dissolved in EtOAc (100 mL) and washed successively with 1 M aqueous HCl (2 × 100 mL), saturated aqueous $NaHCO_3$ (100 mL) and saturated aqueous NaCl (100 mL). The organic phase was isolated, dried (Na_2SO_4), and concentrated to yield crude **11** (4.790 g, 6.86 mmol) in ∼91% yield as a yellow oil. R_f = 0.55 (2:1 PE/EtOAc). Crude **11** (4.790 g, 6.86 mmol) was coevaporated with toluene, dissolved in allylamine (34 mL) and refluxed for 20 h. The reaction mixture was concentrated, dissolved in EtOAc (100 mL), and washed successively with saturated aqueous $NaHCO_3$ (2 × 100 mL) and saturated aqueous NaCl (100 mL). The organic phase was isolated, dried (Na_2SO_4), and concentrated. The residue was purified by flash silica gel chromatography (isocratic 6:1 PE/EtOAc) to produce **12** (2.591 g, 4.60 mmol) in 67% yield as an orange oil. R_f = 0.8 (2:1 PE/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38−7.15 (m, 20H), 5.84−5.70 (m, 1H), 5.12 (dd, J = 1.3, 17.2, 1H), 5.07 (d, J = 10.2, 1H), 4.85 (d, J = 11.1, 1H), 4.80 (d, J = 11.1, 1H), 4.68−4.55 (m, 4H), 4.46 (s, 2H), 3.83 (dd, J = 6.6, 10.2, 1H), 3.76−3.66 (m, 2H), 3.61−3.51 (m, 2H), 3.45−3.33 (m, 2H), 3.18 (dd, J = 6.9, 14.1, 1H), 2.92 (dd, J = 4.5, 11.8, 1H), 2.58−2.53 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.2, 138.7, 138.6, 138.5, 136.2, 129.6, 129.2, 128.6, 128.3, 128.2, 127.9, 127.7, 127.5, 127.4, 127.3, 127.1, 126.7, 117.1, 82.9, 80.1, 78.7, 75.3, 73.2, 72.8, 72.6, 64.6, 59.9, 57.9, 49.1. HRMS: found 564.3090 $[M + H]^+$, calculated for $[C_{37}H_{41}O_4N_1 + H]^+$ 564.3108.

2,3,4,6-Tetra-*O*-benzyl-L-*ido*-1-deoxynojirimycin (13). Potassium *tert*-butoxide (259 mg, 2.3 mmol) was added to a solution of **12** (2.598 g, 4.61 mmol) in DMSO (9.2 mL), and the resulting brown reaction mixture was heated at 100 °C for 30 min. The reaction mixture was charged with 1 M aqueous HCl (9 mL) and stirred vigorously for 15 min. The mixture was poured into saturated aqueous $NaHCO_3$ (100 mL) and extracted with Et_2O (3 × 100 mL). The organic phase was isolated, dried (Na_2SO_4), and concentrated. The residue was purified by flash silica gel chromatography (2:1 → 1:2 PE/EtOAc) to furnish **13** (1.954 g, 3.73 mmol) in 81% yield as a yellow oil. R_f = 0.2 (1:1 PE/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38−7.20 (m, 20H), 4.67−4.50 (m, 8H), 3.67 (dd, J = 9.5, 9.5, 1H), 3.64−3.59 (m, 2H), 3.55 (dd, J = 5.2, 9.5, 1H), 3.44 (dd, J = 6.3, 10.5, 1H), 3.41−3.35 (m, 1H), 3.00 (dd, J = 4.1, 12.9, 1H), 2.86 (dd, J = 6.7, 12.9, 1H), 2.00 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.8, 138.7, 138.6, 138.5, 128.6, 128.5, 128.0, 127.9, 127.8, 127.7, 78.1, 77.3, 77.1, 74.2, 73.5, 72.8, 72.2, 67.4, 54.8, 44.4. HRMS: found 524.2776 $[M + H]^+$, calculated for $[C_{34}H_{37}O_4N_1 + H]^+$ 524.2795.

***N*-[5-(Adamantan-1-ylmethoxy)pentyl]-L-*ido*-1-deoxynojirimycin (4).** A solution of **13** (1.954 g, 3.73 mmol) and **16**[21] (1.029 g, 4.11 mmol) in EtOH/AcOH (93 mL, 9/1, v/v) was purged of oxygen by bubbling argon through the solution. Pd/C (10 wt %, 329 mg) was added to the solution, and the reaction mixture was exposed to 4 bar of hydrogen for 20 h. Removal of Pd/C by filtration over a glass microfiber filter and concentration of the filtrate provided the crude N-alkylated intermediate (R_f = 0.6 in 4:1 PE/EtOAc) as a light-yellow oil. A solution of crude intermediate in EtOH (50 mL) was acidified with 2 M aqueous HCl (12 mL) and purged of oxygen by bubbling argon through the solution. Pd/C (10 wt %, 500 mg) was added to the solution, and the reaction mixture was exposed to 4 bar of hydrogen for 20 h. After removal of Pd/C and concentration, the residue was purified by flash silica gel chromatography (0 → 10% MeOH in $CHCl_3$ + 1% NH_4OH) to afford **4** (1.146 g, 2.88 mmol) in 77% yield over two steps as a white foam. R_f = 0.3 (3:1 $CHCl_3$/MeOH + 1% NH_4OH). ^{1}H NMR (400 MHz, MeOD, major conformation) δ 3.91 (d, J = 5.1, 2H), 3.85 (s, 1H), 3.74 (s, 1H), 3.63 (s, 1H), 3.40 (t, J = 6.2, 2H), 3.31−3.27 (m, 1H), 3.23−2.97 (m, 4H), 2.97 (s, 2H, OCH_2), 1.95 (s, 3H), 1.80−1.59 (m, 10H), 1.56 (d, J = 2.0, 6H), 1.47−1.38 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ 83.2, 72.5, 72.5, 70.2, 64.1, 59.2, 55.4, 53.6, 41.0, 38.5, 35.3, 30.5, 29.9, 25.0. HRMS: found 398.2889 $[M + H]^+$, calculated for $[C_{22}H_{39}O_5N_1 + H]^+$ 398.2901.

Biology. Animals. Experimental procedures were all approved by the appropriate Ethics Committee for Animal Experiments. C57Bl/6J and ob/ob mice (C57Bl/6J background) were obtained from Harlan (Horst, The Netherlands), and ZDF (ZDF/GMi-fa/fa) rats and lean littermates were from Charles River Laboratories (Wilmington, MA). Animals were housed in a light- and temperature-controlled facility. Animals were fed a commercially available lab chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) containing about 6% fat and ∼0.01% cholesterol (w/w). Iminosugars were mixed in the food. In the case of experiments with ZDF rats, compound was administered by oral gavage two times daily.

Plasma and Tissue Sampling. Blood samples were collected by either tail vein or retro-orbital plexus puncture. Animals were sacrificed under isoflurane anesthesia. A large blood sample was collected by cardiac puncture. Tissues were quickly removed and frozen for further analysis.

Analysis of Lipids and Measurement of Enzyme Activities. Lipids were extracted according to Folch et al.[34] Ceramide and glucosylceramide collected from the chloroform phase were determined by HPLC analysis of *o*-phthalaldehyde-conjugated lipids according to a procedure described previously.[35] The chloroform layer was thoroughly dried, and deacylation of

lipids was performed in 0.5 mL of 0.1 M NaOH in methanol in a microwave oven (CEM microwave solids/moisture system SAM-155). After deacylation glycolipids were derivatized on-line for 30 min with *o*-phthalaldehyde. Analysis was performed using an HPLC system (Waters Associates, Milford, MA) and a Hypersil BDS C_{18} 3 μm, 150 mm × 4.6 mm reverse phase column (Alltech). Chromatographic profiles were analyzed using Waters Millennium software. All samples were run in duplicate, and in every run a reference sample was included.

Ganglioside composition was determined by analysis of the acidic glycolipid fraction obtained from the upper phase after Folch extraction. Gangliosides were desalted on a disposable SPE C_{18} column (Bakerbond, Mallinckrodt Baker Inc., Phillipsburg, NJ) and quantified following release of oligosaccharides from gangliosides by ceramide glycanase (recombinant endoglycoceramidase II, Takara Bio Inc., Otsu, Shiga, Japan) digestion. The enzyme was used according to the manufacturer's instructions. Released oligosaccharides were labeled at their reducing end with the fluorescent compound anthranilic acid (2-aminobenzoic acid), prior to analysis using normal-phase high-performance liquid chromatography.[36] IC_{50} values of **2** for various enzyme activities were determined by exposing cells or enzyme preparations to an appropriate range of iminosugar concentrations. IC_{50} values for GCS activity were measured using living cells with NBD-ceramide as substrate.[37] IC_{50} values for the lysosomal GBA1 were measured using 4-methylumbelliferyl-β-D-glucoside as substrate.[38] IC_{50} values for the nonlysosomal glucocerebrosidase (GBA2) were measured with the same substrate as earlier described.[39,40] Lactase, maltase, and sucrase were determined with homogenates of freshly isolated rat or mouse intestine using assay conditions described earlier.[16] Debranching enzyme (α-1,6-glucosidase) was measured with an erythrocyte preparation as enzyme source as described previously.[16]

Analysis of Insulin Signaling in Liver. Livers were quickly collected and lysed in modified RIPA buffer as described earlier.[11] Equal amounts of lysates were separated by SDS−PAGE and immunoblots performed in parallel using anti-pTyr^{1446} IR-β, anti-pSer^{473} AKT, anti-pSer^{2448} mTOR, and anti-p-70S8K (Cell Signaling Technology, Inc.).

Glucose Tolerance Test and Analysis of Sucrose Assimilation. The tolerance test was performed in fasted animals (> 6 h) with oral gavage of glucose (0.5 or 1 g of glucose per kg of body weight). Blood glucose values were measured immediately before and 10, 20, 30, 60, 90, and 120 min after glucose injection. AUCs (areas under the curve, arbitrary units per minute) were determined for individual animals. For analysis of sucrose assimilation, fasted animals (> 6 h) received oral gavage of sucrose (1 g per kg of body weight), and subsequently blood glucose values were measured as described above.

Blood Glucose, HbA1c, and HOMA-IR Analysis. The concentrations of glucose, insulin, and HbA1c levels in blood samples were determined as exactly described previously.[11] For HOMA-IR analysis, the index is from the product of the fasting concentrations of plasma insulin (mU/L) and plasma glucose (mM) divided by 22.5.

Statistical Testing. Values presented in the figures represent the mean ± SEM. Statistical analysis of two groups was assessed by Student's *t* test (two-tailed) or ANOVA for repeated measurement (clamp experiment). Level of significance was set at $p < 0.05$.

Pharmacokinetic Evaluation. Four ZDF/Crl-leprfa male rats were administered a single 3 mg/kg intravenous dose of either **2** or **4** in normal saline. In another group four ZDF/Crl-leprfa male rats were administered a single 10 mg/kg oral dose of either **2** or **4** in normal saline. Each animal was dosed following an overnight fast. Following dose administration, whole blood samples were collected via the jugular vein catheter from each animal for up to 12 h. Blood samples were processed to plasma and analyzed by liquid chromatography with tandem mass spectrometry. Pharmacokinetic parameters were determined using standard noncompartmental methods by WinNolinTM, version 5.1.

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Supporting Information Available: PK profiles in ZDF rats of **2** and **4**; experimental procedures, characterization data, and ^{1}H- and ^{13}C-APT NMR spectra for iminosugars **3**, **4**, **8**, **14**, **15**, **20**−**22**, **27**−**29**, and their synthetic intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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