Design and Synthesis of Novel *N*-Acetylgalactosamine-Terminated Glycolipids for Targeting of Lipoproteins to the Hepatic Asialoglycoprotein Receptor

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A novel glycolipid has been prepared that contains a cluster glycoside with an unusually high affinity for the asialoglycoprotein receptor (ASGPr) and a bile acid moiety that mediates stable incorporation into lipidic particles. The glycolipid spontaneously associated with low-density lipoproteins (LDL) and high-density lipoproteins (HDL) within human and murine plasma, and loading of lipoproteins with this glycolipid resulted in an efficient dose-dependent recognition and uptake of LDL and HDL by the liver (and not by spleen) upon intravenous injection into wild-type mice. Preinjection with asialoorosomucoid largely inhibited the uptake, establishing that both HDL and LDL were selectively recognized and processed by the ASGPr on liver parenchymal cells. Finally, repeated intravenous administration of the glycolipid to hyperlipidemic LDL receptor-deficient mice evoked an efficient and persistent cholesterol-lowering effect. These results indicate that the glycolipid may be a promising alternative for the treatment of hyperlipidemic patients who do not respond sufficiently to current cholesterol-lowering therapies.

Introduction

Clinical data indicate a positive correlation between LDL cholesterol levels and the occurrence of arteriosclerosis,[1,2] while a strong inverse correlation has been demonstrated between HDL cholesterol levels and cardiovascular disease.[3,4] However, recent insights indicate that the antiatherogenic properties of HDL are not dictated by its plasma levels, but rather by its functionality (i.e. capability to deliver cholesteryl esters to the liver). In fact, increasing the flux of HDL to the liver may also be atheroprotective, as it has been shown that overexpression of the hepatic HDL receptor SR-BI in heterozygous LDLr-deficient ($ldlr^{-/-}$) mice on a high fat/high cholesterol diet resulted in strong reduction in plasma HDL cholesterol and concomitantly reduced atherosclerotic lesion area.[5,6] Thus, these studies clearly illustrate that stimulation of the hepatic uptake of LDL and HDL will be an effective entry to antiatherosclerotic therapy and led us to explore the feasibility to induce effective clearance of these lipoproteins via the asialoglycoprotein receptor (ASPGr).[7]

The ASGPr is a high-capacity receptor uniquely expressed on the surface of hepatocytes to mediate the hepatic uptake and subsequent lysosomal processing of galactose (Gal)- and *N*-acetylgalactosamine (GalNAc)-terminated substrates from the serum,[8] at which the affinity for GalNAc is approximately 50-fold higher than for Gal.[9] In addition to the ASGPr on hepatocytes, a second receptor recognizing galactose and fucose groups is present in the liver on Kupffer cells (galactose particle receptor, galactose/fucose receptor)[10,11] and preferentially recognizes a high density of either fucose or galactose on either proteins[11−13] or particles.[14,15] Previous proof-of-concept studies already showed that the bifunctional glycolipid TG(20Å)C (glycolipid **1**, Figure 1), consisting of a cholesteryl moiety for anchorage to lipoproteins and a triantennary Gal-terminated glycoside with moderate affinity for the ASGPr, was able to associate with LDL and HDL and induce their liver uptake in rodents. However, due to the relatively high hydrophilicity of the glycolipid, rapid exchange of the glycolipid occurred in the blood, leading to only a moderate 20% liver uptake of these lipoproteins after preloading with TG(20Å)C.[16] To stabilize incorporation of the glycolipid into lipoproteins, the lipophilicity of the anchoring moiety was increased by adding a fatty acid chain to the steroid core structure. In addition, the intrinsically labile methylene acetals connecting the glycol spacers to the tris cluster core in TG(20Å)C were replaced by more stable ether bonds.[17] The resulting glycolipid compound (glycolipid **2**, Figure 1) indeed showed a very stable interaction with lipidic particles which was retained in the blood and resulted in an effective targeting of liposomes to the ASGPr on hepatocytes.[17,18] However, a major setback still was that high glycolipid surface densities resulted in elimination of liposomes by the galactose particle receptor,[17,18] and preliminary data showed that glycolipid **2** redirected LDL almost exclusively to Kupffer cells.

In this paper, we introduce an improved amphiphilic glycolipid, **3** (see Figure 1), in which, with respect to **2**, two important modifications are present. A tyrosine moiety was introduced to allow for trace labeling with ^{125}I, providing the possibility to perform kinetic studies in vivo. In addition, the galactose residues within the triantennary cluster glycoside were replaced by GalNAc

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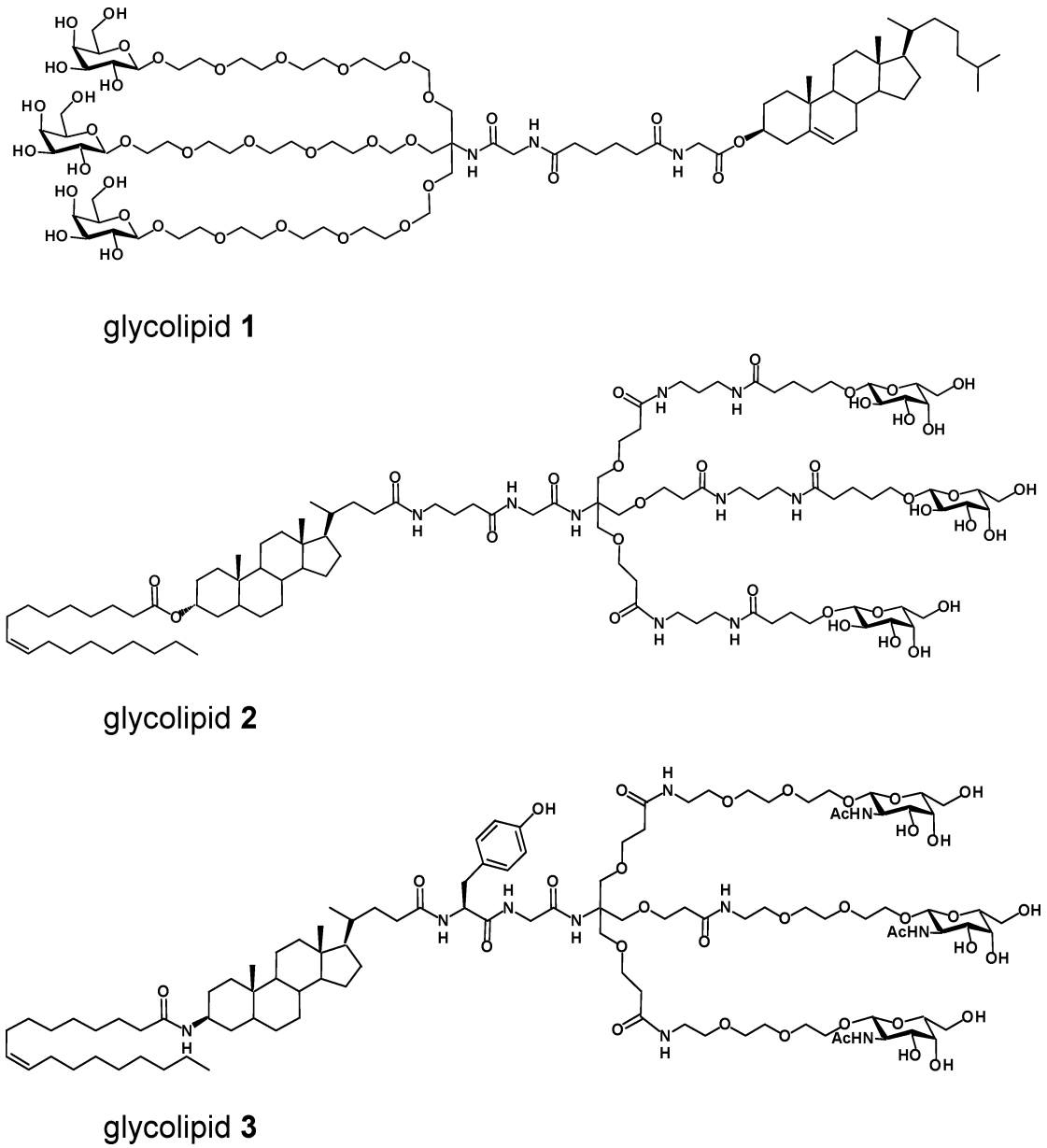


Figure 1. Chemical structures of glycolipids TG(20Å)C (**1**), (3α(oleoyloxy)-5β-cholanoyl)-GABA-Gly-tris(Gal)$_3$ (**2**), and (3β-(oleoylamido)-5β-cholanoyl)-Tyr-Gly-tris(GalNAc)$_3$ (**3**).

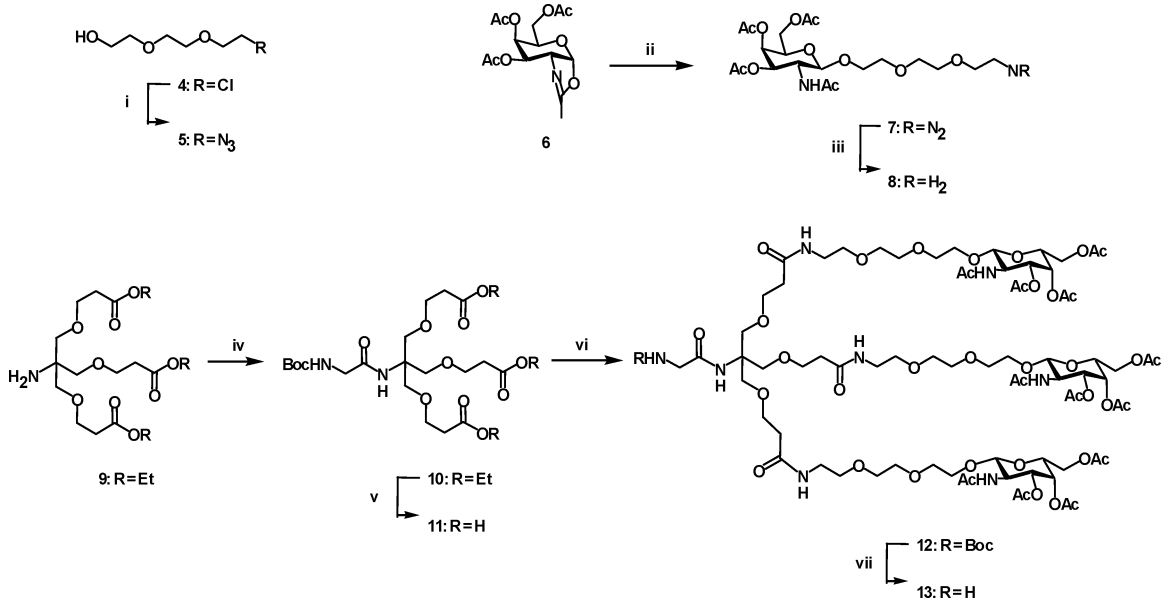
residues, which led to a 50-fold increased affinity of the cluster glycoside toward the ASGPr on hepatocytes (K_i 2 nM).[18] The resulting compound was tested for its ability to associate with plasma lipoproteins and to induce their liver uptake and for its cholesterol-lowering capacity in hyperlipidemic *ldlr*$^{-/-}$ mice. We conclude from these data that glycolipid **3** is very effective in promoting cholesterol transport to hepatocytes and thus may be a promising alternative for hyperlipidemic individuals who do not respond sufficiently to current cholesterol-lowering therapies, such as familial hypercholesterolemic patients.[19,20]

Results and Discussion

Synthesis. Previously, we synthesized glycolipid **1**, which only modestly induced the hepatic uptake of lipoproteins, as its cholesterol moiety afforded an only weak association with lipoproteins.[16,21] Replacement of cholesterol by the highly lipophilic lithocholic oleate (LCO) residue yielded glycolipid **2**, which showed a markedly improved association with lipidic particles, but the galactose-exposing cluster glycoside still induced particle uptake by Kupffer and splenic cells via the galactose particle receptor depending on substrate size and substrate surface density of the glycolipid.[17] Previous studies by us[22,23] and others[24] have established that triantennary GalNAc-containing ligands displayed a higher affinity for the ASGPr than the corresponding diantennary GalNAcs or triantennary galactosides. Therefore, we now synthesized a third-generation glycolipid by combining the lipophilic LCO unit possessing high affinity for lipidic particles with a triantennary GalNAc-containing glycoside (glycolipid **3**). This novel triantennary glycoside with 20 Å-spaced terminal GalNAc residues appeared to display a low nanomolar affinity for the ASGPr (2 nM) and to redirect differently sized stable unilamellar liposomes to hepatocytes in vitro and in vivo.[18]

In this study we describe the synthesis of this novel glycolipid **3** and present new biological data indicating

Scheme 1. Synthesis of the Cluster Galactoside Synthon 13[a]



[a] Reagents: (i) NaN_3, NaI, 62%; (ii) **5**, TMSOTf, 58%; (iii) PPh_3, THF, 49%; (iv) BocGlyOH, BOP, DiPEA, 93%; (v) aq NaOH, dioxane, 100%; (vi) **8**, HOBt, PyBOP, DiPEA, DMF; (vii) TFA/DCM 1/4, 100%.

that this improved glycolipid is able to direct both LDL and HDL to the ASGPr on hepatocytes in vivo, thereby avoiding lipoprotein uptake by the galactose particle receptor on Kupffer cells and spleen. In analogy to glycolipid **1**, we have attached the GalNAc units to the tris core by a flexible ethylene glycol linker to warrant an optimal orientation and thus recognition of the terminal sugar moieties by the ASGPr. Given this galactosamine synthon terminus at each dendrimer arm, an eight-atom spacer moiety sufficed to establish a 20 Å long connection between the separate GalNAcs and the tris branching point. Thus, 2-(2-(2-chloroethoxy)ethoxy)ethanol **4** was converted to its corresponding azido-derivative **5** and the galactosamine building-unit **8** was synthesized by glycosylation of the known[25] oxazoline **6** with derivative **5** (see Scheme 1). Condensation in the presence of trimethylsilyl triflate (TMSOTf) gave compound **7** in good yield, while subsequent reduction of the azido moiety with triphenylphosphine afforded the terminal amine derivative **8**.

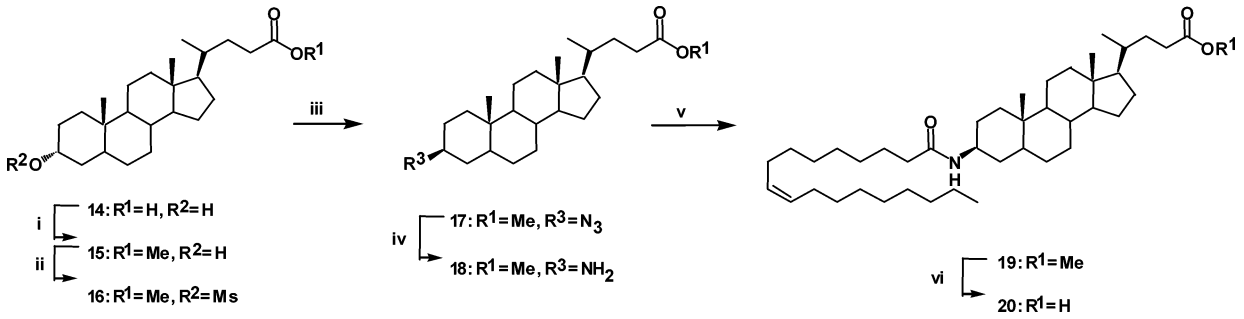
The triantennary core-unit, which has been described earlier,[17] was smoothly functionalized by a *N*-Boc-glycine under standard amino acid coupling conditions to furnish compound **10**. Hydrolysis of the terminal ethyl esters rendered the trivalent carboxylic acid **11**. Purified **11** was condensed with excess of GalNAc **8** in order to afford compound **12**. Removal of the Boc-protecting group with TFA furnished the trivalent galactosamine **13** with a free amine, which allowed functionalization of the cluster with a tyrosine moiety. The incorporation of this amino acid was performed to enable the ready introduction of a ^{125}I radiolabel and thus to enable us track the incorporation efficacy and pharmacokinetics of the glycolipid per se. Thus, Fmoc-protected tyrosine(OtBu) was reacted with amine **13** under the agency of TBTU and HOBt to afford compound **21**. Subsequent treatment with piperidine (20% in DMF) provided galactoside **22**.

Having the required key synthon **22** in hand, attention was focused on the construction of the lipophilic component of the target glycolipid. Lithocholic acid was converted into its methyl ester **15**, followed by the introduction of a mesyl protecting group at the 3-hydroxyl position. The mesylated methyl ester was reacted with sodium azide to afford, by a nucleophilic substitution reaction, the required azido derivative **17** under inversion of the stereochemistry. Reduction of the azide group readily furnished amine **18**. Freshly distilled oleoyl chloride was used to acylate the free amino group of compound **18**, followed by saponification of the methyl ester to furnish 3-oleoylamidolithocholic acid, synthon **20** (Scheme 2).

The bile oleoylamido acid **20** was joined in moderate yield to the cluster GalNAc via TBTU/HOBt-aided coupling. Deprotection of the fully protected compound **23** was achieved in two steps. The *tert*-butyl ester was removed from the tyrosine by acid treament, while deprotection of the acetyl groups of GalNAc was accomplished by incubation with sodium methanolate. Purification of the target compound was performed by gel filtration over Sephacryl S100 in methanol. The homogeneity and identity of **3** was fully confirmed by NMR spectroscopy and mass spectrometry (Scheme 3). Glycolipid **3** forms stable micelles with a slightly larger size than HDL (8−10 nm) as judged from size exclusion chromatography on a Superose 6 column.[18]

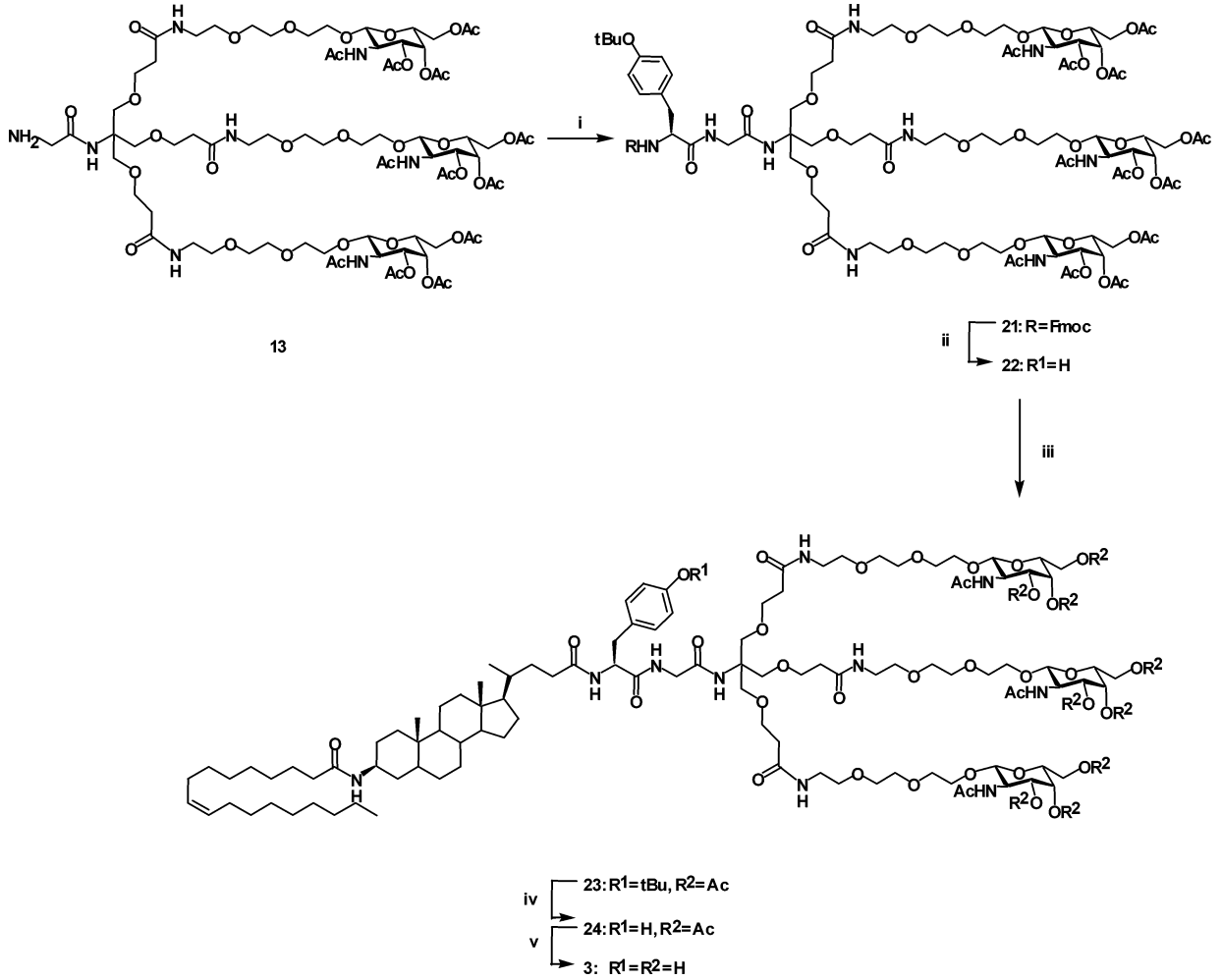
Association of Glycolipids with Lipoproteins. Previously, we showed by FPLC that the presently applied hydrophobic bile acid residue **20** (see Scheme 2) mediates rapid incorporation of glycolipids **2**[17] and **3**[18] into liposomes by intercalation into the liposomal phospholipid bilayer. We now demonstrate using agarose gel electrophoresis that glycolipid **3** avidly and quantitatively incorporated into HDL and LDL at all glycolipid concentrations tested, leading to a reduced electrophoretic mobility of the lipoproteins by shielding off their negative surface charge (Figure 2). Since glycolipid **3** incorporates instantaneously and quantitatively into lipoproteins, even without the need for incubation at 37 °C (not shown), it indeed has a very

Scheme 2. Synthesis of the Lipophilic Anchor Synthon 20[a]



[a] Reagents: (i) MeOH, HCl, 100%; (ii) MsCl, pyridine; (iii) NaN_3, DMF, 70% two steps; (iv) PPh_3, THF; (v) oleoyl chloride, Et_3N, DCE, 86%; (iv) NaOH, dioxane, H_2O, 100%.

Scheme 3. Synthesis of Glycolipid 3[a]



[a] Reagents: (i) FmocTyr(tBu)OH, TBTU, HOBt, DiPEA, DMF, 70%; (ii) 20% piperidine/DMF, 75%; (iii) **20**, TBTU, HOBt, DiPEA, DMF, 80%; (iv) DCM/TFA, 100%; (v) NaOMe, MeOH, 90%.

high affinity for lipoproteins. Since glycolipid **2** induced similar effects on LDL and HDL (not shown), the orientation of the oleoyl moiety relative to the steroid skeleton (α for glycolipid **2** and β for glycolipid **3**) apparently does not affect the distribution of glycolipid over LDL and HDL. Incorporation of the glycolipid did not significantly alter the lipoprotein size, as judged from Sepharose 6 elution profiles (not shown). At the applied amounts of lipoproteins (i.e. equal protein weight of HDL and LDL), the glycolipid displayed an increased association with LDL. This enhanced binding to LDL probably reflects the presence of a larger total phospholipid surface area (i.e. 1.7-fold) of LDL versus HDL at the applied particle ratio. Therefore, the glycolipid does not show preference for association with either lipoprotein. Indeed, the glycolipid induced a similar dose-dependent reduction in the mobility of both lipoproteins, and the relative distribution of the glycolipid over HDL and LDL remained constant over the whole glycolipid concentration range (Figure 2). At the highest amount tested, glycolipid **3** did not affect the composition of LDL (27.2 $\pm$ 1.7% protein, 3.3 $\pm$ 0.3% triglycerides, 18.8 $\pm$ 1.5% phosphatidylcholine, 42.1 $\pm$ 1.4% cholesteryl esters, and 8.5 $\pm$ 0.2% free cholesterol)

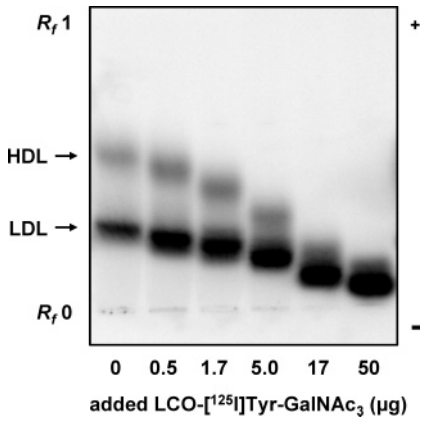


Figure 2. Association of the glycolipid with isolated lipoproteins. Glycolipid **3** (0−50 μg) mixed with a trace amount of [^{125}I]glycolipid **3** (20 ng) was incubated with HDL and LDL (5 μg of protein each) for 30 min at 37 °C. The mixtures were subjected to agarose gel electrophoresis, and radioactivity was visualized. +, anode; -, cathode; R_f 0, position application slots; R_f 1, position front marker bromophenol blue.

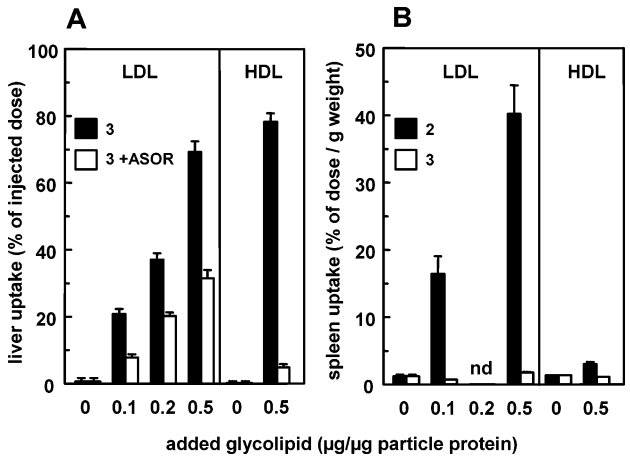


Figure 3. Liver uptake of control and glycolipid-laden lipoproteins in mice. [^{125}I]LDL (5 μg of protein) or [^{3}H]HDL (10 μg of protein) were incubated with the indicated amounts of glycolipid **3** (A, B) or **2** (B) and injected into anaesthetized C57Bl/6J mice, without or with previous injection of ASOR (25 mg/kg) at 1 min before injection of the lipoproteins (A). At 10 min after injection, liver lobules (A) and spleen (B) were taken, and the amount of radioactivity was determined. Values are means ± variation of two experiments.

or HDL (54.9 ± 1.3% protein, 2.2 ± 0.4% triglycerides, 22.9 ± 1.2% phosphatidylcholine, 17.7 ± 1.0% cholesteryl esters, and 2.4 ± 0.4% free cholesterol), as determined by incubation of isolated lipoproteins with the glycolipid and subsequent reisolation of lipoproteins by density gradient ultracentrifugation.

Liver Uptake of Glycolipid/Lipoprotein Complexes. We have recently shown that the novel triantennary GalNAc-terminated cluster glycoside **13** has a 50-fold increased affinity toward the ASGPr on hepatocytes as compared to triantennary galactose-terminated glycosides (K_i 2 vs 100 nM).[18] We now evaluated the effect of the increased affinity of glycolipids **3** over **2** on the induction of the hepatic uptake of lipoproteins. Upon intravenous injection into C57BL/6J mice, the hepatic uptake of [^{125}I]LDL and [^{3}H]HDL was negligible (<1% of the dose at 10 min after injection) (Figure 3A). Prior incubation of LDL or HDL with the novel glycolipid **3** caused a dose-dependently enhanced liver uptake, reaching 70 ± 3% and 78 ± 2% of the dose, respectively, at 0.5 μg of glycolipid per μg of particle protein (Figure 3A), similar to the effect of glycolipid **2** on the hepatic clearance of both lipoproteins. At this glycolipid loading, the ASGPr-specific inhibitor ASOR[26] inhibited the glycolipid-induced hepatic clearance of LDL and HDL for 63% and 94%, respectively. In contrast, the enhanced liver uptake of glycolipid **2**-laden LDL could not be inhibited by ASOR (not shown). These data indicate that the triantennary galactose moiety of glycolipid **2** mediates the uptake of LDL exclusively via the galactose particle receptor on liver macrophages (i.e. Kupffer cells), while replacement of the galactose groups by GalNAcs within the novel glycolipid **3** results in prominent uptake of both HDL and LDL by the ASGPr on hepatocytes. This observation is of major importance for application of glycolipids in cholesterol-lowering therapy, since only hepatocytes are able to directly excrete cholesterol from the body via the bile. Also, whereas glycolipid **2** enhanced the association of LDL with the spleen by 33.5-fold, glycolipid **3** did not affect the splenic uptake (and extrahepatic accumulation in general) of both lipoproteins (Figure 3B). The application of glycolipid **3** in cholesterol-lowering therapy will thus not result in unwanted cholesterol delivery to tissues other than the liver.

We have previously observed that a high surface density of glycolipid **2** leads to a shift in uptake of liposomes from hepatocytes to Kupffer cells,[17] whereas glycolipid **3** induced the uptake of liposomes by hepatocytes, irrespective of its surface density.[18] A similar phenomenon may underlie the different effect of the glycolipids on the intrahepatic distribution of LDL over hepatocytes and Kupffer cells. Whereas glycolipids can evenly distribute over the phospholipid surface of liposomes, the surface of LDL is largely covered by its protein constituent apolipoprotein B-100,[27,28] leaving relatively small clefts for the association of glycolipid. Therefore, the glycolipids will accumulate in these clefts, resulting in a local high glycolipid density, which, for glycolipid **2**, may lead to the induced LDL uptake by Kupffer cells. In contrast, the affinity of glycolipid **3** for the ASGPr is sufficiently high and selective to still induce the uptake of LDL by hepatocytes.

Association of Glycolipid with Lipoproteins In Vivo. To evaluate whether glycolipid **3** is able to spontaneously associate with plasma lipoproteins upon injection prior to elimination by the ASGPr, the tyrosine moiety of glycolipid **3** was radioiodinated, which allowed for kinetic studies in mice. The serum decay of glycolipid **3** upon iv injection (3.33 mg/kg) in C57BL/6J mice was relatively slow ($t_{1/2}$ ~30 min) and could be mainly attributed to a gradually increasing uptake by the liver (44 ± 3% at 60 min after injection) (Figure 4A). At 60 min after injection, glycolipid **3** was almost completely (>95%) associated with HDL (1.063 < d < 1.21 g/mL), as can be appreciated from the overlapping [^{125}I]glycolipid and HDL-cholesterol profiles (Figure 4B). Apparently, the affinity of the bile acid anchoring moiety of the glycolipid for lipoproteins is sufficiently high to overcome rapid elimination of the glycolipid by the liver. Instead, the glycolipid selectively accumulated in lipoproteins, which thus enables the subsequent delivery of lipoproteins to hepatocytes.

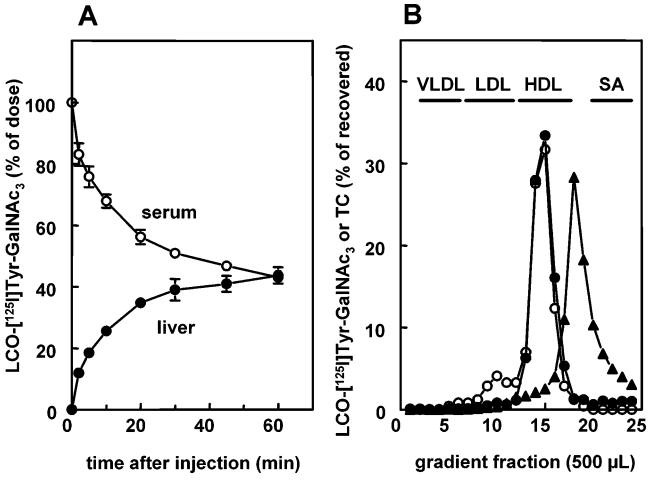


Figure 4. Association of the glycolipid with lipoproteins in plasma in vivo. [^{125}I]Glycolipid **3** (75 μg) was injected into the vena cava inferior of anaesthetized C57Bl/6J mice, and the liver uptake and serum decay were determined. Values are means ± variation of two experiments (A). At 60 min after injection, the mice were bled, and serum samples (100 μL; circles) or pure [^{125}I]glycolipid **3** (5 μg; triangles) were subjected to density gradient ultracentrifugation. The gradients were subdivided from top (fraction 1) to bottom (fraction 24), and the fractions were assayed for ^{125}I-activity (●, ▲) and total cholesterol (TC) (○) (B).

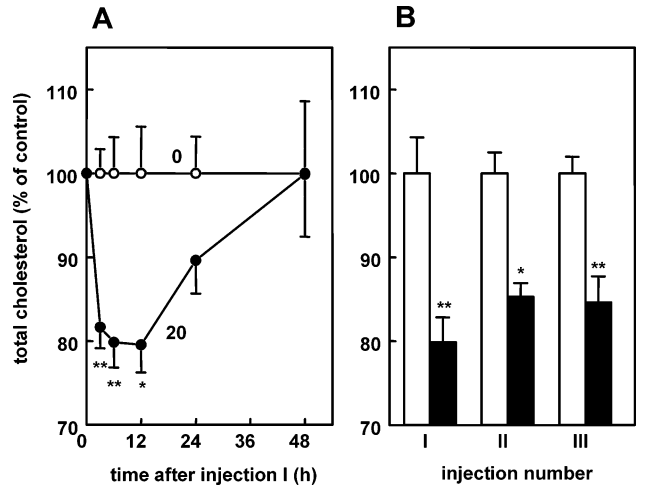


Figure 5. Hypocholesterolemic effect of the glycolipid in mice. Glycolipid **3** (20 mg/kg; ●) or PBS (○) was injected via the tail vein into *ldlr*$^{-/-}$ mice, and plasma samples were taken at the indicated times (A). Subsequently, mice received two additional injections of glycolipid **3** (closed bars) or PBS (open bars) at 72 h intervals, and additional plasma samples were taken at 6 h after injection (B). Total cholesterol levels in the plasmas were determined and expressed as percentage of control values. The initial cholesterol values (100%) in plasma samples taken before injection of glycolipids were 37.9 ± 3.6 mmol/L. Values are means ± SEM (n = 6). *P < 0.05, **P < 0.01

Cholesterol-Lowering Effect of Glycolipids. Since glycolipid **3** appeared superior to glycolipid **2** with respect to its ability to target lipoproteins specifically to hepatocytes, the ability of glycolipid **3** to reduce plasma cholesterol (i.e. lipoprotein) levels was evaluated in hyperlipidemic *ldlr*$^{-/-}$ mice as a model of familial hypercholesterolemia. Upon iv injection of glycolipid **3** into *ldlr*$^{-/-}$ mice, a marked lipoprotein-lowering effect was detected (Figure 5A). The effect peaked already at 3−6 h (20%; P < 0.01) and was still significant (P < 0.05) up to 12 h after injection. Baseline levels were reached only after 48 h after injection. For clinical application of glycolipids in hyperlipidemia, glycolipids should be able to show consistent effects upon repeated administration. Importantly, three consecutive injections of glycolipid **3** mice at 72 h time intervals resulted in consistent reductions in total cholesterol levels (Figure 5B). The glycolipid showed no sign of toxicity at the applied dose as compared to saline injection. At 3 or 24 h after iv injection into mice, no effects were observed on the weight of several organs (liver, heart, kidneys, spleen) and plasma levels of enzymes that would indicate systemic toxicity (lactate dehydrogenase) or liver toxicity (alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase). In addition, the glycolipid did not cause hemolysis after incubation with heparinized blood at concentrations equivalent to doses of 100 mg/kg (not shown).

In conclusion, we have prepared glycolipid **3** with an unusually high affinity and specificity for the hepatic ASGPr. The glycolipid is capable of associating with plasma lipoproteins and thereby induces the uptake of LDL and HDL by hepatocytes, which are able to secrete excess cholesterol from the body via the bile as neutral sterols and bile acids, rather than by Kupffer and splenic cells. In this respect, the glycolipid presented here is superior to the latest glycolipid introduced by our group for hepatocyte-directed targeting purposes.[17] Importantly, injection of glycolipid **3** resulted in a prolonged and repeated cholesterol-lowering in a mouse model of familial hypercholesterolemia. This advantageous property of glycolipid **3** opens the way for the employment of glycolipids for cholesterol-lowering therapy in those hypercholesterolemic patients who do not respond to conventional cholesterol-lowering therapies.

Experimental Section

General. Pyridine, *N,N*-dimethylformamide (DMF), 1,2-dichloroethane (DCE), dichloromethane (DCM), 1,4-dioxane, ethanol, tetrahydrofuran (THF), toluene, *N,N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), piperidine, triethylamine (Et_3N), and methanol were from Biosolve (Valkenswaard, The Netherlands). Methanol was stored over 3 Å molecular sieves, and DMF, DCE, DCM, and THF were stored over 4 Å molecular sieves. Trimethylsilyl trifluoromethanesulfonate (TMSOTf, Fluka), triphenylphosphine (Acros), *N*-Boc-L-glycine (NovaBiochem), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, NovaBiochem), 1-hydroxybenzotriazole (HOBt, NovaBiochem), *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU, NovaBiochem), *N*-Fmoc-*O-tert*-butyl-L-tyrosine (NovaBiochem); mesyl chloride (Merck); sodium azide (Acros); and 1,3-dipropanedithiol (Aldrich) were used as received. Before use oleoyl chloride (Fluka) was distilled under reduced pressure to afford a colorless oil. [1α,2α-^{3}H]cholesteryl oleate and ^{125}I (carrier-free) were from Amersham Biosciences (Little Chalfont, UK). Asialoorosomucoid (ASOR) was prepared by enzymatic desialylation (approximately 70%, as judged by the extent of sialic acid release) of human α_1-acid glycoprotein (orosomucoid) from Cohn Fraction VI (99%; Sigma) as described.[29] All other chemicals were of analytical grade. Merck Kieselgel 60 F_{254} DC Alufolien was used for TLC analysis. Compounds were visualized with UV light (254 nm). Carbohydrate compounds were visualized by charring with sulfuric acid/ethanol (1/4, v/v) at ∼150 °C and bile acid esters[30] by charring with $MnCl_2$ at ∼150 °C. Compounds containing NH groups were visualized after treatment of the plates with chlorine, spraying with a solution of TDM,[31] and subsequent heating to ∼150 °C or by spraying with a solution of ninhydrine in acetic acid/water and subsequent heating. Column

chromatography was performed with Kieselgel 60, 230−400 mesh (Merck). Gel filtration was performed with Sephadex LH-20 (Pharmacia). $^{13}C\{^1H\}$ NMR spectra (75 MHz) and ^1H NMR spectra (300 MHz) were recorded using a Bruker WM-300 spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Fast atom bombardment (FAB) mass spectrometry was carried out using a JEOL JMS SX/SX102A four-sector mass spectrometer, coupled to a JEOL MS-MP7000 data system.

2-(2-(2-Azidoethoxy)ethoxy)ethanol (5). 2-(2-(2-Chloroethoxy)ethoxy)ethanol **4** (10 g, 59.3 mmol) was dissolved in dry ethanol (50 mL), and sodium iodide (0.9 g, 6 mmol) and sodium azide (4.55 g, 70 mmol; preactivated with hydrazine monohydrate) were added. The resulting mixture was refluxed for 5 days until TLC analysis (DCM/methanol 9/1, v/v) showed one product. The reaction mixture was filtered (salts) and concentrated. The residue was dissolved in DCM (50 mL) and stored for 16 h at 4 °C. After filtration and concentration, compound **5** was obtained as clear oil. Yield: 6.42 g (36.6 mmol, 62%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 50.0 (CH_2−N_3), 60.6 (CH_2OH), 69.2, 69.5, 69.8 (3 × CH_2O). ^1H NMR ($CDCl_3$): δ 3.44 (m, 2H, CH_2N_3), 3.68 (m, 10H, CH_2O).

2-(2-(2-Azidoethoxy)ethoxy)ethyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-galactopyranoside (7). The known oxazoline **6**[25] (1.04 g, 3.0 mmol) and 2-(2-(2-azidoethoxy)-ethoxy)ethanol **5** (0.80 g, 4.6 mmol) were dissolved in 1,2-dichloroethane (DCE) (10 mL). Molecular sieves (4 Å) were added and the mixture was stirred for 30 min. Then, TMSOTf[23] (0.27 mL, 1.5 mmol) was added, and the reaction was stirred overnight. According to TLC analysis (DCM/methanol 9/1, v/v), the oxazoline **6** was completely converted into a lower running product. The mixture was filtered over Hyflo. The filtrate was taken up in DCM (50 mL), washed with aq $NaHCO_3$ (1 M, 50 mL) and water (50 mL), dried over $MgSO_4$, and concentrated to an oil. The crude product was purified by silica gel column chromatography, with DCM/methanol (1/0 → 94/6, v/v) as eluent. Yield: 1.29 g (2.56 mmol, 85%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 20.3 (CH_3 Ac), 22.8 (CH_3 NHAc), 50.2 (C-2 Gal), 50.3 (CH_2−N_3), 61.1 (CH_2O), 61.3 (C-6 Gal), 68.2−72.2 (4 × CH_2O), 66.4, 70.2, 70.5 (C-3,4,5 Gal), 101.7 (C-1 Gal), 169.7, 170.1 (C=O). ^1H NMR ($CDCl_3$): δ 1.99, 2.00, 2.05 (3 × s, 9H, CH_3, Ac), 2.16 (s, 3H, CH_3, NHAc), 3.48 (t, 2H, CH_2N_3), 3.61−3.76 (m, 8H, CH_2O), 3.89 (m, 1H, H-5 Gal), 3.90 (t, 2H, CH_2O), 4.15 (t, 2H, H-6a, H-6b Gal), 4.22 (m, 1H, H-2 Gal, J_{N-2} = 9.3 Hz), 4.78 (d, 1H, H-1 Gal, J_{1-2} = 8.6 Hz), 5.06 (dd, 1H, H-3 Gal, J_{3-4} = 3.3 Hz, J_{3-2} = 11.2 Hz), 5.32 (d, 1H, H-4 Gal), 6.15 (d, 1H, N*H*).

2-(2-(2-Aminoethoxy)ethoxy)ethyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-galactopyranoside (8). The azide **7** (314 mg, 622 μmol) was dissolved in THF (10 mL), and triphenylphosphine (196 mg, 746 μmol) was added. The mixture was stirred for 48 h, until TLC analysis (toluene/methanol 1/1, v/v) showed the absence of starting material. Water (32 μL, 1.8 mmol) was added to the reaction, and stirring was continued for another 24 h. TLC analysis (toluene/methanol 1/1, v/v and 2-propanol/water 4/1, v/v) showed one product that was both H_2SO_4- and ninhydrine-positive. Trifluoroacetic acid (TFA, 96 μL, 1.25 mmol) and toluene (10 mL) were added, the mixture was concentrated to near dryness and finally coevaporated with toluene (2 × 10 mL). The crude amine **8** thus obtained was used immediately for the synthesis of the protected cluster **12**. $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 20.2 (CH_3 Ac), 22.6 (CH_3 NHAc), 42.4 (CH_2NH_2), 49.9 (C-2 Gal), 61.3 (C-6 Gal), 68.5−70.2 (CH_2O), 66.5, 69.9 (C-3,4,5 Gal), 101.1 (C-1 Gal), 169.6, 169.8, 169.9 (C=O), 170.9 (C=O, NHAc). ^1H NMR ($CDCl_3$): δ 1.88, 1.94, 2.03 (3 × s, 9H, CH_3, Ac), 2.09 (s, 3H, CH_3, NHAc), 3.20 (m, 2H, CH_2NH_2), 3.47 (m, 4H, 2 × CH_2O), 3.61 (t, 2H, CH_2O), 3.66 (t, 2H, CH_2O), 3.75 (m, 2H, CH_2O), 3.91 (m, 2H, H-5 Gal, N*H*), 4.11 (m, 3H, H-2, H-6a, H-6b Gal), 4.87 (d, 1H, H-1 Gal, J_{1-2} = 8.5 Hz), 5.20 (dd, 1H, H-3 Gal, J_{3-4} = 3.3 Hz, J_{3-2} = 11.2 Hz), 5.34 (d, 1H, H-4 Gal).

***N*-(*tert*-butoxycarbonylglycinyl)tris(carboxyethoxymethyl)aminomethane triethyl ester (10).** Tris(carboxyethoxyethyl)aminoethane triethyl ester **9**[17] (729 mg, 1.73 mmol), *N*-*tert*-butoxycarbonyl-glycine (*N*-BocGlyOH) (306 mg, 1.73 mmol), and BOP (920 mg, 2.08 mmol) were dissolved in DCE (15 mL), and acylation was started by addition of DIPEA (0.72 mL, 4.15 mmol). The resulting mixture was stirred overnight at 20 °C. According to TLC analysis (DCM/methanol 9/1, v/v), the reaction was complete. The mixture was taken up in DCM (50 mL); washed with H_2O (50 mL), $NaHCO_3$ (1 M, 50 mL), and brine (50 mL); dried ($MgSO_4$); and concentrated. The resulting oil was applied to a silica gel column, using ethyl acetate/hexane (1/2 → 1/0, v/v) as eluent, affording compound **10**. Yield: 0.90 g (1.61 mmol, 93%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 14.0 (CH_3 OEt), 28.1 (CH_3 Boc), 34.7 (CH_2C=O), 44.2 (CH_2 Gly), 59.6 (C_q tris), 60.2 (OCH_2CH_3), 66.6 (OCH_2), 68.9 (CH_2 tris), 80.5 (C_q Boc), 156.4 (C=O Boc), 169.1 (C=O ester), 171.4 (C=O amide). ^1H NMR ($CDCl_3$): δ 1.26 (t, 9H, CH_3 ethyl), 1.45 (s, 9H, CH_3 Boc), 2.03 (d, 2H, CH_2 Gly, J_{H-NH} = 0.6 Hz), 2.53 (t, 6H, CH_2C=O, J = 6.2 Hz), 3.70 (m, 12 H, OCH_2), 4.13 (dq, 6H, OCH_2, J = 7.1 Hz), 5.38 (bs, 1H, N*H* Boc), 6.39 (d, 1H, N*H* Gly).

***N*-(*tert*-butoxycarbonylglycinyl)tris(carboxyethoxymethyl)aminomethane (11).** The triester **10** (0.85 g, 1.52 mmol) was dissolved in a mixture of 1,4-dioxane (90 mL) and water (27 mL), and aqueous NaOH (4 M, 3 mL) was added. After stirring for 16 h, TLC analysis (DCM/methanol/acetic acid 18/2/1, v/v/v) showed complete conversion of the starting material into one product. The mixture was carefully neutralized by the addition of Dowex (50 W x 8, H^+ form). The mixture was filtered, and the filtrate concentrated. The residue was coevaporated with toluene (2 × 10 mL) and purified by means of a silica gel column using DCM/methanol/acetic acid (100/0/1 → 95/5/1, v/v/v) as eluent. Yield: 0.75 g (1.52 mmol, 100%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 28.7 (CH_3 Boc), 36.7 (CH_2C=O), 44.9 (CH_2 Gly), 61.2 (C_q tris), 68.2 (OCH_2), 70.0 (CH_2 tris), 80.7 (C_q Boc), 156.5 (C=O Boc), 172.2 (C=O amide), 176.0, 176.3 (C=O, carboxyl). ^1H NMR ($CDCl_3$): δ 1.44 (s, 9H, CH_3 Boc), 2.09 (s, 2H, CH_2 Gly), 2.57 (t, 6H, CH_2C=O), 3.71 (bs, 14 H, OCH_2), 6.59 (bs, 1H, N*H* Gly).

***N*-(*tert*-butoxycarbonylglycinyl)tris{(1-[2-(2-aminoethoxy)ethoxy]ethoxy-2-acetamido-3,4,6-tetra-*O*-acetyl-2-deoxy-*β*-D-galactosamine)(carboxyethoxymethyl)}methane (12).** To a solution of the tricarboxylate **11** (74 mg, 149 μmol) and the crude amine **8** (<622 μmol) in DMF (5 mL) were added DIPEA (188 μL, 1.08 mmol), HOBt (73 mg, 0.54 mmol), and TBTU (173 mg, 0.54 mmol). The mixture was stirred overnight, after which an extra amount of DIPEA (188 μL, 1.08 mmol) was added and the mixture was stirred again for another 18 h. According to TLC analysis (DCM/methanol 4/1, v/v), the reaction was complete. The reaction mixture was taken up in DCM (40 mL); washed with dilute H_3PO_4 (1 M, 40 mL), aq $NaHCO_3$ (1 M, 40 mL), and water (40 mL); dried ($MgSO_4$); and concentrated to an oil. This oil was applied to a silica gel column, and elution was performed with DCM/methanol (1/0 → 84/16, v/v). The crude product thus obtained was further purified by Sephadex LH20 gel filtration, using DCM/methanol (1/1, v/v) as eluent. Yield: 161 mg (86 μmol, 58%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 20.3 (CH_3 Ac), 22.7 (CH_3 NHAc), 27.9 (CH_3 Boc), 34.1, 36.1, 38.8 (CH_2C=O), 43.1 (CH_2 Gly), 50.2 (C-2 Gal), 51.3 (CH_2−NH), 59.4 (CH_2O), 61.2 (C-6 Gal), 66.4, 70.1 (C-3,4,5 Gal), 67.0 ⁻ 70.0 (CH_2O), 79.2 (C_q, Boc), 101.0 (C-1 Gal), 155.7 (C=O Boc), 169.5, 169.9, 170.6 (C=O ester, amide), 171.3 (C=O NHAc). ^1H NMR ($CDCl_3$): δ 1.44 (s, 9H, CH_3 Boc), 1.96, 1.99, 2.05 (3 × s, 27H, CH_3 Ac), 2.09 (s, 2H, CH_2 Gly), 2.15 (s, 9H, CH_3 NHAc), 2.43, 2.55 (m, 6H, CH_2C=O), 2.97, 3.05, 3.25 (m, 6H, CH_2NH), 3.46⁻3.94 (m, 24H, OCH_2, H-5 Gal), 4.13 (m, 9H, H-6a, H-6b, H-2 Gal), 4.79 (d, 3H, H-1 Gal), 5.21 (dd, 3H, H-3 Gal), 5.34 (d, 3H, H-4 Gal, $J_{4,3}$ = 3.0 Hz).

Glycinyltris{(1-[2-(2-aminoethoxy)ethoxy]ethoxy-2-acetamido-3,4,6-tetra-*O*-acetyl-2-deoxy-*β*-D-galactosamine)-(carboxyethoxymethyl)}methane (13). The fully protected derivative **12** (155 mg, 83 μmol) was dissolved in TFA/DCM (1/4, v/v; 5 mL). After the mixture was stirred for 60 min, toluene (20 mL) was added. The mixture was concentrated and the residue coevaporated with toluene (3 × 10 mL). According

to TLC analysis (DCM/methanol 9/1, v/v), the starting material was completely converted into a lower running product. The thus obtained crude **13** was used without prior purification for the synthesis of compound **21**. $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 20.2 (*C*H_3 Ac), 22.5 (*C*H_3 NHAc), 34.3, 35.9, 38.9 (*C*H_2C=O), 44.7 (*C*H_2 Gly), 50.1 (C-2 Gal), 51.4 (*C*H_2−N_3), 60.2, 66.4, 67.0, 68.5, 69.8 (*C*H_2O), 61.3 (C-6 Gal), 66.5, 70.3 (C-3,4,5 Gal), 101.2 (C-1 Gal), 170.2, 170.4 (*C*=O ester, amide), 171.9 (*C*=O NHAc).

Lithocholic Acid Methyl Ester (15).[32] Lithocholic acid (3.77 g, 10 mmol) was dissolved in methanol (25 mL). Concentrated HCl (0.5 mL, 36−38%) was added, and the solution was heated at reflux temperature (65 °C). After 30 min, TLC analysis (toluene/ethanol 85/15, v/v; $MnCl_2$ staining) showed that the ester formation was complete. The reaction mixture was cooled overnight at 0 °C. The crystallized solid was collected by filtration and washed with cold methanol (25 mL). The white solid **15** was dried at room temperature. Yield: 2.97 g (7.6 mmol, 76%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 11.9 (*C*H_3 C18), 18.1 (*C*H_3 C21), 20.7 (*C*H_2), 23.3 (*C*H_3 C19), 24.1, 26.3, 27.1, 28.0, 30.4, 30.8, 30.9, 34.4, 35.3 (*C*H_2), 35.2 (*C*H C20), 35.7 (*C*H C8), 40.0 (C_q C10), 40.3 (*C*H C9), 42.0 (*C*H C5), 42.6 (C_q C13), 51.4 (O*C*H3), 55.8 (*C*H C17), 56.3 (*C*H C14), 71.6 (C*H*OH C3), 174.7 (*C*=O C24). ^1H NMR ($CDCl_3$): δ 0.64 (s, 3H, C*H*$_3$ C18), 0.90 (s, 3H, C*H*$_3$ C21), 0.92 (s, 3H, C*H*$_3$ C19), 0.97−1.98 (m, 27H, C*H*, C*H*$_2$), 2.28 (m, 2H, C*H*$_2$C=O C23), 3.33 (bs, 1H, O*H*), 3.66 (s, 3H, OC*H*$_3$).

3-Azidolithocholic Acid Methyl Ester (17). Lithocholic acid methyl ester **15** (0.78 g, 2.0 mmol) was dissolved in DCE (10 mL) and concentrated. Next, the methyl ester was dissolved in pyridine (20 mL) and cooled to 0 °C, and following addition of mesyl chloride (186 μL, 2.4 mmol), the resulting mixture was allowed to warm to room temperature. After incubation for 3 h, TLC analysis (toluene/ethanol 85/15, v/v; $MnCl_2$ staining) showed complete disappearance of the starting compound **15**. The reaction mixture was concentrated and coevaporated with toluene (3 × 10 mL). The intermediate compound **16** was dissolved in DMF (20 mL), sodium azide (156 mg, 2.4 mmol) was added, and the mixture was subsequently heated overnight at 100 °C. The reaction mixture was concentrated in vacuo and taken up in DCM (30 mL). The organic layer was washed with water (20 mL), $NaHCO_3$ (1 M, 20 mL), and brine (20 mL); dried ($MgSO_4$); and concentrated. The crude brown solid was recrystallized from DCM/hexane, affording a white solid. Yield: 0.67 g (1.62 mmol, 81%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 11.8 (*C*H_3 C18), 18.1 (*C*H_3 C21), 20.8 (*C*H_2), 23.6 (*C*H_3, C19), 23.9, 24.5, 26.1, 26.3, 28.0, 30.0, 30.5, 30.8, 34.7 (*C*H_2), 35.1 (*C*H C20), 35.4 (*C*H C8), 37.1 (*C*H, C9), 39.9 (*C*H C5), 40.0 (C_q C10), 42.5 (C_q C13), 51.2 (*C*H, C17), 55.8 (*C*H C14), 56.4 (O*C*H3), 58.5 (*C*H C3 C*H*-N_3), 174.4 (*C*=O C24). ^1H NMR ($CDCl_3$): δ 0.65 (s, 3H, C*H*$_3$ C18), 0.90 (d, 3H, C*H*$_3$ C21 J = 6.4 Hz), 0.95 (s, 3H, C*H*$_3$ C19), 1.03−2.00 (m, 27H, C*H*, C*H*$_2$), 2.28 (m, 2H, C*H*$_2$C=O C23), 3.66 (s, 3H, OC*H*$_3$).

3-Aminolithocholic Acid Methyl Ester (18). The azido derivative **17** (0.15 g, 0.36 mmol) was dissolved in methanol (1.8 mL) to a concentration of 0.2 M. Et_3N (0.25 mL, 1.8 mmol) and 1.3-propanedithiol (0.18 mL, 1.8 mmol) were added, and the resulting mixture was heated at 60 °C for 2 days. TLC analysis (hexane/diethyl ether 3/1, v/v) established that the conversion was complete. After addition of toluene (2 mL) the reaction mixture was concentrated and coevaporated with toluene (3 × 10 mL), resulting in a yellowish solid. The crude product was purified by means of silica gel chromatography using hexane/diethyl ether (5/1 → 1/1, v/v) as eluent, affording **18** as a white solid. Yield: 101 mg (0.26 mmol, 72%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 11.7 (*C*H_3 C18), 18.0 (*C*H_3 C21), 20.8 (*C*H_2), 23.6 (*C*H_3 C19), 23.9, 26.0, 26.4, 27.5, 28.9, 29.6, 30.7, 33.2, 34.7, 36.6 (*C*H_2), 35.0 (*C*H C20), 35.3 (*C*H C8), 36.2 (*C*H C9), 39.5 (*C*H C5), 39.9 (C_q C10), 42.4 (C_q C13), 51.1 (O*C*H3), 55.7 (*C*H C17), 56.3 (*C*H C14), 66.5 (*C*H C3 C*H*-NH_2), 174.4 (*C*=O C24).

3-Oleoylamidolithocholic Acid Methyl Ester (19). The amine **18** (156 mg, 400 μmol) was dissolved in dry DCE (10 mL). The solution was cooled to 0 °C, and triethylamine (88 μL, 0.60 mmol) and oleoyl chloride (0.20 mL, 0.60 mmol) were added. After the mixture had been stirred for 3 h, TLC analysis (hexane/diethyl ether 2/3, v/v) revealed complete conversion of starting material. The reaction mixture was taken up in DCM (40 mL), washed with aq $NaHCO_3$ (1 M, 40 mL) and water (40 mL), dried ($MgSO_4$), and concentrated to an oil. This oil was applied to a silica gel column, and elution was performed with hexane/diethyl ether (4/1 → 3/1, v/v). Yield: 225 mg (344 μmol, 86%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 11.7 (*C*H_3 C18), 13.7 (*C*H_3 oleoyl), 17.9 (*C*H_3 C21), 20.8 (*C*H_2), 23.5 (*C*H_3 C19), 22.4, 23.9, 24.7, 24.8, 25.9, 26.2, 26.9, 27.8, 28.8, 28.9, 29.1, 29.3, 29.4, 29.5, 30.4, 30.5, 30.7, 31.6, 34.3, 34.5 (*C*H_2), 35.0 (*C*H C20), 35.4 (*C*H C8), 37.1 (*C*H C9), 39.6 (*C*H C5), 39.9 (C_q C10), 42.4 (C_q C13), 50.8 (O*C*H3), 55.7 (*C*H, C17), 56.2 (*C*H C14), 69.8 (*C*H C3 C*H*-NH), 129.3, 129.5 (C=*C*H, oleoyl), 172.4, 173.7 (*C*=O). ^1H NMR ($CDCl_3$): δ 0.65 (s, 3H, C*H*$_3$ C18), 0.83−0.92 (m, 6H, C*H*$_3$ C21, oleoyl, 0.96 (s, 3H, C*H*$_3$ C19), 1.08−1.16 (m, 8H, C*H*$_2$), 1.26−1.43 (m, 30H, C*H*$_2$), 1.52−1.73 (m, 8H, C*H*$_2$), 1.75−2.05 (m, 7H, C*H*), 2.17−2.38 (m, 4H, C*H*$_2$C=O), 3.66 (s, 3H, OC*H*$_3$), 5.07 (bs, 1H, N*H*), 5.34 (m, 2H, C=C*H* oleoyl).

3-Oleoylamidolithocholic Acid (20). The methyl ester **19** (200 mg, 306 μmol) was dissolved in a mixture of 1,4-dioxane (30 mL) and water (9 mL), and deprotection was started by adding aq NaOH (4 M, 1 mL). The mixture was stirred overnight. TLC analysis (hexane/diethyl ether 2/3, v/v) revealed that the starting material was completely converted into a product with zero mobility. The mixture was carefully neutralized by the addition of Dowex (50 W x 8, H^+ form) and then filtered. The filtrate was concentrated to a small volume and applied to a silica gel column. Elution with hexane/diethyl ether (1/0 → 1/1, v/v) furnished pure **20** as a white solid. Yield: 196 mg (306 μmol, >99%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 12.0 (*C*H_3 C18), 14.1 (*C*H_3 oleoyl), 18.2 (*C*H_3 C21), 20.8 (*C*H_2), 23.3 (*C*H_3 C19), 22.7, 24.2, 24.7, 25.1, 26.3, 26.7, 27.0, 27.2, 28.1, 29.1, 29.3, 29.5, 29.7, 30.8, 31.0, 31.9, 32.3, 34.0, 34.6, 34.8, 35.0 (*C*H_2), 35.3 (*C*H C20), 35.8 (*C*H C8), 40.1 (C_q C10), 40.4 (*C*H, C9), 41.9 (*C*H C5), 42.7 (C_q C13), 56.0 (*C*H, C17), 56.5 (*C*H C14), 74.1 (*C*H C3 C*H*-NH), 129.7, 130.0 (C=*C*H oleoyl), 179.9, 180.4 (*C*=O). ^1H NMR ($CDCl_3$): δ 0.65 (s, 3H, C*H*$_3$ C18), 0.86−0.91 (m, 9H, C*H*$_3$), 1.07−1.11 (m, 8H, C*H*$_2$), 1.26−1.30 (m, 30H, C*H*$_2$), 1.61−1.63 (m, 8H, C*H*$_2$), 1.80−1.83 (m, 2H, C*H*), 2.00−2.05 (m, 5H, C*H*), 2.23−2.37 (m, 4H, C*H*$_2$C=O), 5.35 (m, 2H, C=C*H* oleoyl).

***O-tert*-Butyl-*N*−Fmoc-tyrosinyl-glycinyl-tris{(1-[2-(2-aminoethoxy)ethoxy]ethox y-2-acetamido-3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-galactosamine)(carboxyethoxymethyl)}methane (21).** The crude amine **13** (155 mg, <83 μmol) and *N*-FmocTyr(tBu)OH (57 mg, 125 μmol) were dissolved in DMF (10 mL). To the solution were added DIPEA (30 μL, 170 μmol), HOBT (17 mg, 125 μmol), and TBTU (40 mg, 125 μmol), and the mixture was stirred for 3 h until TLC analysis (DCM/methanol 85/15, v/v) indicated the reaction to be complete. The reaction mixture was taken up in DCM (40 mL); washed with dilute H_3PO_4 (1 M, 40 mL), aq $NaHCO_3$ (1 M, 40 mL), and water (40 mL); dried ($MgSO_4$); and concentrated to an oil. This oil was applied to a silica gel column, and elution was performed with DCM/methanol (1/0 → 88/12, v/v), followed by Sephadex LH-20 gel filtration, using DCM/methanol (1/1, v/v) as eluent. Yield: 129 mg (58 μmol, 70%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 20.4 (*C*H_3 Ac), 22.8 (*C*H_3 NHAc), 28.5 (*C*H_3 tBu), 34.4, 36.4, 39.0 (*C*H_2C=O), 37.7 (*C*H_2 Tyr), 43.1 (*C*H_2 Gly), 50.3 (C-2 Gal), 51.6 (*C*H_2−NH), 56.2 (*C*H Tyr), 59.9 (*C*H_2O), 61.4 (C-6 Gal), 66.7, 70.4 (C-3,4,5 Gal), 66.9−70.3 (*C*H_2O), 79.2 (C_q tBu), 101.4 (C-1 Gal), 119.8, 124.9, 126.9, 127.6 (*C*H Fmoc), 124.0, 129.6 (*C*H Tyr), 131.3, 154.1 (C_q Tyr Fmoc), 141.1, 143.6 (C_q Fmoc), 155.9 (*C*=O Fmoc), 170.3, 170.5, 170.6 (*C*=O ester, amide), 171.6 (*C*=O NHAc). ^1H NMR ($CDCl_3$): δ 1.28 (s, 9H, C*H*$_3$ tBu), 1.94, 1.98, 2.02 (3 × s, 27H, C*H*$_3$ Ac), 2.07 (s, 2H, C*H*$_2$ Gly), 2.13 (s, 9H, C*H*$_3$ NHAc), 2.41−2.53 (m, 6H, C*H*$_2$C=O), 2.92−3.05 (m, 6H, C*H*$_2$NH), 3.14 (m, 2H, βC*H*$_2$ Tyr), 3.59−3.92 (m, 45H, OC*H*$_2$, H-5 Gal), 4.14 (m, 9H, H-6a, H-6b, H-2 Gal), 4.36 (m, 2H, αC*H* Tyr, C*H* Fmoc), 4.49 (m, 2H, OC*H*$_2$ Fmoc), 4.74 (d, 3H, H-1 Gal J = 8.3 Hz), 5.18 (dd, 3H, H-3 Gal

J_{3-2} = 10.5 Hz), 5.33 (d, 3H, H-4 Gal), 6.86−7.11 (4H, C*H* Tyr), 7.30−7.76 (8H, C*H* Fmoc).

***O-tert*-Butyl-*N*-tyrosinylglycinyltris{(1-[2-(2-aminoethoxy)ethoxy]ethoxy-2-acetamido-3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-galactosamine)(carboxyethoxymethyl)}methane (22).** The fully protected derivative **21** (120 mg, 54 μmol) was dissolved in piperidine/THF (5/95, v/v; 10 mL) and the mixture was stirred for 1 h. TLC analysis (DCM/methanol 85/15, v/v) revealed complete conversion of starting material into a single ninhydrine-positive product with lower mobility. Toluene (10 mL) was added, the mixture was concentrated to a small volume and coevaporated with toluene (2 × 10 mL). The residue was purified by means of silica gel column using DCM/methanol (100/1 → 8/2) as eluent. Yield: 81 mg (41 μmol, 75%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 20.4 (*C*H_3 Ac), 22.9 (*C*H_3 NHAc), 28.5 (*C*H_3 tBu), 34.3, 36.2, 38.9 (*C*H_2C=O), 37.3 (*C*H_2 Tyr), 44.3 (*C*H_2 Gly), 50.2 (C-2 Gal), 51.4 (*C*H_2−NH), 56.1 (*C*H Tyr), 59.8 (*C*H_2O), 61.3 (C-6 Gal), 67.1, 70.2 (C-3,4,5 Gal), 66.5−69.9 (*C*H_2O), 78.1 (C_q tBu), 101.2 (C-1 Gal), 124.0, 124.9, (*C*H Tyr), 131.3, 153.9 (C_q Tyr), 170.1, 170.2, 170.8 (*C*=O ester, amide), 171.5 (*C*=O, NHAc). ^1H NMR ($CDCl_3$): δ 1.34 (s, 9H, C*H*$_3$ tBu), 1.96, 1.99, 2.04 (3 × s, 27H, C*H*$_3$ Ac), 2.09 (s, 2H, C*H*$_2$ Gly), 2.14 (s, 9H, C*H*$_3$ NHAc), 2.35−2.58 (m, 6H, C*H*$_2$C=O), 2.92−3.05 (m, 6H, C*H*$_2$NH), 3.25 (m, 2H, βC*H*$_2$ Tyr), 3.57−4.16 (m, 54H, OC*H*$_2$, H-5, H-6a, H-6b, H-2 Gal), 4.23 (m, 1H, αC*H* Tyr), 4.80 (m, 3H, H-1 Gal), 5.20 (m, 3H, H-3 Gal), 5.35 (m, 3H, H-4 Gal), 6.93 (d, 2H C*H* Tyr), 7.12 (d, 2H, C*H* Tyr).

3-Oleoylamidolithocholic Acid [*O-tert*-Butyl-*N*-tyrosinylglycinyltris{(1-[2-(2-aminoethoxy)ethoxy]ethoxy-2-acetamido-3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-galactosamine)(carboxyethoxymethyl)}methane] (23). Amine **22** (75 mg, 38 μmol) and acid **20** (36 mg, 57 μmol) were dissolved in DMF (5 mL), and acylation was commenced by addition of DIPEA (14 μL, 80 μmol), HOBT (8 mg, 56 μmol), and TBTU (18 mg, 56 μmol). The mixture was stirred overnight after which TLC analysis (DCM/methanol 85/15, v/v) showed that the reaction has completed. The reaction mixture was taken up in DCM (40 mL); washed with dilute H_3PO_4 (1 M, 40 mL), aq $NaHCO_3$ (1 M, 40 mL), and water (40 mL); dried ($MgSO_4$); and concentrated to an oil. This oil was applied to a silica gel column, and elution was performed with DCM/methanol (1/0 → 88/12, v/v). The crude product thus obtained was further purified by Sephadex LH20 gel filtration, using DCM/methanol (1/1, v/v) as eluent. Yield: 80 mg (30 μmol, 80%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 11.7 (C18 LCO), 13.8 (CH_3 oleoyl), 18.0 (C21 LCO), 20.3 (CH_3, Ac), 20.7 (CH_2 LCO), 22.8 (CH_3, NHAc), 23.5 (C19 CH LCO), 23.8, 24.8, 25.3, 25.8, 26.1, 26.8, 27.8 (CH_2 LCO), 28.5 (CH_3 tBu), 28.7, 28.8, 28.9, 29.1, 29.3, 29.4, 30.4, 31.3, 31.5, 32.8 (CH_2 LCO), 34.5, 36.2, 38.8 (CH_2C=O), 35.1 (C20 CH LCO), 35.3 (C8 CH LCO), 37.2 (CH_2 Tyr), 39.5 (C9 CH LCO), 39.8 (C10 C_q LCO), 42.4 (C13 C_q LCO), 43.1 (CH_2 Gly), 50.2 (C-2 Gal), 51.3 (CH_2−NH), 55.5 (C17 CH LCO), 55.8 (CH, Tyr), 56.2 (C14 CH LCO), 59.7, 60.2 (CH_2O), 61.3 (C-6 Gal), 66.5, 70.0, 70.2 (C-3,4,5 Gal), 67.1−70.1 (CH_2O), 77.9 (C_q tBu), 101.2 (C-1 Gal), 123.8, 129.4, 129.6 (CH Tyr, C=CH oleoyl), 131.3, 153.8 (C_q Tyr), 170.0, 170.1, 170.6, 171.4, 173.0 (C=O). ^1H NMR ($CDCl_3$): δ 0.63 (s, 3H, C18), 0.88 (s, 3H, CH_3 oleoyl), 0.89 (d, 3H, C21 J = 6.6 Hz), 0.96 (s, 3H, C19), 1.13−1.63 (m, 53H, CH, CH_2 LCO), 1.28 (s, 9H, CH_3 tBu), 1.95, 1.99, 2.04 (3 × s, 27H, CH_3 Ac), 2.08 (s, 2H, CH_2 Gly), 2.14 (s, 9H, CH_3 NHAc), 2.27−2.54 (m, 10H, CH_2C=O), 2.93−3.44 (m, 8H, CH_2NH, βCH_2 Tyr), 3.62−3.95 (m, 45H, OCH_2, H-5 Gal), 4.13−4.19 (m, 9H, H-6a, H-6b, H-2), 4.77 (m, 3H, H-1 Gal J_{1-2} = 8.3 Hz), 5.16 (dd, 3H, H-3 Gal J_{3-4} = 2.2 Hz, J_{3-2} = 11.3 Hz), 5.35 (m, 5H, C=CH LCO, H-4 Gal), 6.90 (d, 2H CH Tyr), 7.09 (d, 2H, CH Tyr).

3-Oleoylamidolithocholic Acid [Tyrosinylglycinyltris{(1-[2-(2-aminoethoxy)ethoxy]ethoxy-2-acetamido-2-deoxy-β-D-galactosamine)(carboxyethoxymethyl)}methane] (3). The fully protected glycolipid **23** (32 mg, 12 μmol) was dissolved in DCM/TFA (4/1, v/v; 5 mL). The mixture was stirred for 1 h, when TLC analysis (DCM/methanol 85/15, v/v) revealed the reaction to be complete. The mixture was diluted with toluene (10 mL), concentrated to a small volume, and coevaporated with toluene (2 × 10 mL). The crude product **24** was applied to a silica gel column, using DCM/methanol (1/0 → 82/18, v/v) as eluent. Yield: 27 mg (11 μmol, 91%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 11.8 (C18 LCO), 13.9 (CH_3 oleoyl), 18.0 (C21 LCO), 20.4 (CH_3 Ac), 22.4 (CH_3 NHAc), 23.7 (C19 CH LCO), 23.9, 24.9, 25.4, 25.9, 26.3, 27.0, 27.9, 28.9, 29.1, 29.3, 29.5, 30.5, 31.7, 32.9 (CH_2 LCO), 34.6, 36.1, 39.3 (CH_2C=O), 35.4 (C20 CH LCO), 37.2 (C8 CH LCO), 37.9 (CH Tyr), 39.6 (C9 CH LCO), 39.9 (C10 C_q LCO), 42.5 (CH_2 Gly), 43.1 (C13 C_q LCO), 50.7 (C-2 Gal), 51.6 (CH_2−NH), 54.7, 55.6, 56.3 (C14, C17 CH LCO, CH Tyr), 57.9, 60.1, 60.5 (CH_2O), 61.4 (C-6 Gal), 66.5, 70.4 (C-3,4,5 Gal), 63.4−70.2 (CH_2O), 101.2 (C-1 Gal), 115.4 (CH Tyr), 129.5, 129.8, 130.1 (CH Tyr, C=CH oleoyl), 155.7, 159.0 (C_q Tyr), 170.3, 170.5, 172.6, 172.9, 173.5 (C=O).

Compound **24** was dissolved in methanolic sodium methanolate (0.05 M, 2 mL). After overnight stirring, TLC analysis (DCM/methanol 85/15, v/v and 2-propanol/25% NH_4OH 1/1, v/v) showed complete conversion of the starting material into a single product (compound **3**). The crude deprotected product thus obtained was purified using a Sephacryl S100 gel filtration column, with water as eluent. The product fractions were pooled, concentrated, and lyophilized. Yield: 8.2 mg (3.8 μmol, 32% based on **23**). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 12.2 (C18 LCO), 14.2 (*C*H_3 oleoyl), 18.4 (C21 LCO), 23.0 (*C*H_3 NHAc), 24.1 (C19 *C*H LCO), 21.9, 22.9, 25.4, 26.4, 26.8, 27.4, 28.4, 29.3, 29.5, 29.9, 30.0, 31.0, 32.1, 33.4 (*C*H_2 LCO), 34.0, 35.6, 36.9, 40.4 (*C*H_2C=O), 35.8 (C20 *C*H LCO), 35.9 (C8 *C*H LCO), 38.0 (*C*H Tyr), 40.2 (C9 *C*H LCO), 41.5 (*C*H_2, Gly), 41.9 (C10 C_q LCO), 43.5 (C13 C_q LCO), 52.2 (C-2 Gal), 54.3 (*C*H_2−NH), 56.2 (C17 *C*H LCO), 57.4 (*C*H Tyr), 57.9 (C14 *C*H LCO), 60.5 (*C*H_2O), 61.7 (C-6 Gal), 68.6, 72.7, 75.3 (C-3,4,5 Gal), 67.0−70.8 (*C*H_2O), 101.8 (C-1 Gal), 115.6 (*C*H Tyr), 127.8 (C_q Tyr), 129.9, 130.2 (C=*C*H oleoyl), 130.5 (*C*H Tyr), 156.1 (C_q Tyr), 169.8, 172.9, 173.4, 174.4, 175.2 (*C*=O).^1H NMR ($CDCl_3$): δ 0.65 (s, 3H, C18), 0.87−1.61 (m, 63H, C*H*$_3$, C*H*$_2$ LCO), 2.01 (s, 9H, C*H*$_3$, NHAc), 2.33 (t, 3H, C*H*$_3$ LCO), 2.43−2.55 (m, 8H, C*H*$_2$C=O), 2.93−3.27 (m, 6H, C*H*$_2$NH), 3.34−3.83 (48H, OC*H*$_2$, Gal), 4.43 (d, 3H, H-1 Gal, J = 8.4 Hz), 4.54 (m, 3H, H-5 Gal), 5.05 (m, 3H, H-3 Gal), 5.33 (m, 5H, H-4 Gal, C=C*H* LCO), 6.69 (d, 2H C*H* Tyr), 7.04 (d, 2H, C*H* Tyr). Mass (FAB^+): *m/e* 2206.6 [M + Na]$^+$.

Animals. Male C57Bl/6J mice (12−14 week old, 24−28 g) were obtained from Broekman Instituut BV (Someren, The Netherlands) and fed ad libitum with standard rat/mouse chow (SRM-A, Hope Farms). Homozygous LDL-receptor deficient ($ldlr^{-/-}$) mice have originally been obtained from the Jackson Laboratory (Bar Harbor, ME) and are maintained as a colony at our local housing facility at the Gorlaeus Laboratory. Three weeks before use, female $ldlr^{-/-}$ mice (20 week old, 25−30 g) were fed a Western-type diet (Hope Farms) ad libitum for three weeks. All experiments were approved by the Ethics Committee on Animal Experiments of the University of Leiden.

Isolation of Lipoproteins. Human LDL and HDL were isolated from freshly isolated serum of healthy volunteers as described[33] and dialyzed at 4 °C against PBS, 1 mmol/L EDTA, pH 7.4. Protein concentrations were determined according to Lowry et al.[34] using BSA as a standard.

Glycolipid. The freeze-dried glycolipids **2** and **3** were dissolved in phosphate-buffered saline (PBS) at a final concentration of 50 μg/μL and stored at −80 °C under Argon. Their stability was routinely checked by TLC (*n*-butanol/*n*-propanol/25% NH_4OH/H_2O = 15/40/30/15, v/v/v/v) and subsequent staining for cholesterol ($MnCl_2$) and sugar (H_2SO_4) moieties.

Radiolabeling of Lipoproteins and Glycolipids. HDL was labeled with [^{3}H]cholesteryl oleate (∼25 dpm/ng protein) and LDL was radioiodinated and purified as described,[35] while glycolipid **3** was radiolabeled by the Iodogen method using a Iodogen (10 μg)-coated reaction tube. More than 97% of the radiolabel in [^{125}I]TC-LDL was 10% TCA precipitable, and the specific ^{125}I-activity was ∼150 dpm/ng of protein. [^{125}I]glycolipid **3** (1300 dpm/ng) migrated as a single band (R_f 0.64) on TLC (*n*-butanol/*n*-propanol/25% NH_4OH/H_2O = 15/40/30/15, v/v/v/v).

Association of Glycolipids with Lipoproteins. Human HDL and LDL (5 μg of protein each) were incubated (30 min

at 37 °C) with ^{125}I-labeled or unlabeled glycolipid **3** in PBS, pH 7.4. The mixtures were electrophoresed on a 0.75% (w/w) agarose gel at pH 8.8, and the gels were fixed and dried. Lipids were visualized by Sudan Black and ^{125}I-activity was monitored using a Packard Instant Imager (Hewlett-Packard Co., Palo Alto, CA).

Kinetic Studies in Mice. Mice were anesthetized,[17] and the abdomens were opened. [^{125}I]glycolipid **3** or radiolabeled lipoproteins, previously incubated with PBS or glycolipid **3** (30 min at 37 °C), were injected via the inferior vena cava. Blood and liver samples were taken and processed as previously described.[36] When indicated, mice received a preinjection of ASOR (25 mg/kg) at 1 min before injection of the lipoproteins. At 60 min after injection of [^{125}I]glycolipid **3**, mice were bled, and 100 μL serum samples were subjected to density gradient ultracentrifugation.[33] Tubes were fractionated (24 × 0.5 mL) using a Multiprobe 104DT Robotic System (Packard Instrument Co.), and fractions were assayed for ^{125}I-activity and for cholesterol using the Roche Molecular Biochemicals enzymatic kit for cholesterol.

Cholesterol-Lowering Effect of Glycolipids. PBS or glycolipid **3** (20 mg/kg) was injected via the tail vein into conscious *ldlr*$^{-/-}$ mice. Blood samples (25 μL) were taken from the tail vein by heparin-coated capillaries, and the plasmas were analyzed for total cholesterol as described above. Alternatively, mice were sacrificed at 3 or 24 h after injection, and the weight of several organs was determined. Plasma levels of lactate dehydrogenase (LDH), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), and γ-glutamyl transferase (γGT) were determined using SYS-3 BM/Hitachi 747 kits from Roche Molecular Biochemicals.

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