Design and Synthesis of Novel Amphiphilic Dendritic Galactosides for Selective Targeting of Liposomes to the Hepatic Asialoglycoprotein Receptor

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A series of glycolipids have been prepared which contain a cluster galactoside moiety with high affinity for the hepatic asialoglycoprotein receptor and a bile acid ester moiety which mediates stable incorporation into liposomes. Loading of liposomes with these glycolipids at a ratio of 5% (w/w) resulted in efficient recognition and uptake of the liposomes by the liver. Preinjection with asialofetuin almost completely inhibited the uptake, establishing that the liposomes were selectively recognized and processed by the asialoglycoprotein receptor on liver parenchymal cells. In contrast, a glycolipid content of 50% (w/w) led to a liver uptake that could not be inhibited by preinjection with asialofetuin, indicating that the liposomes were now processed by the Gal/Fuc-recognizing receptor on liver macrophages. The results presented in this study are important for future targeting of water-soluble and amphiphilic drugs, enveloped in these glycolipid-laden liposomes, to parenchymal liver cells.

Introduction

Parenchymal liver cells express a unique receptor protein, the galactose (Gal)- or *N*-acetylgalactosamine (GalNAc)-recognizing asialoglycoprotein receptor (ASGPr)[1]. In view of its exclusive and abundant presence on parenchymal cells, and because of its high rate of internalization, the ASGPr was considered a promising candidate target in many drug carrier studies.[2−4] Since 1984, a number of well-defined glycopeptides,[5−11] displaying nanomolar affinity for the ASGPr, have been developed and used successfully for this purpose. Uptake of DNA and antisense oligodeoxynucleotides (ODNs) by parenchymal liver cells could be realized in vitro and in vivo[12−14] after direct coupling to the synthetic low molecular weight glycopeptides. However, the coupling of a homing device to a universal carrier such as liposomes can offer a number of advantages as compared to a direct covalent attachment of the homing device to the drug. First, encapsulation of the drug by the lipid bilayer of the liposome protects the drug from early degradation, and prevents interference of the drug with the ligand recognition process. Second, the use of these vesicles allows the targeting of both hydrophobic and water-soluble drugs without the need of prior modification. Third, a more favorable drug-to-carrier ratio may be attained. Fourth, the absence of a covalent linker between carrier and drug greatly facilitates the intracellular release of the drug from the lysosomal compartment. These advantageous properties have prompted us[15−17] and others[18−22] to study the design, synthesis, and targeting potential of bifunctional glycolipids consisting of a lipophilic anchor moiety for incorporation into drug-containing liposomes and a multivalent galactoside moiety for conferring high-affinity recognition by the ASGPr. It was shown by Spanjer et al.[16] that association of a cholesterylated cluster galactoside (tris-gal-chol) with liposomes induces receptor-mediated uptake in the liver of these liposomes. However, analysis of the intrahepatic cellular distribution revealed that tris-gal-chol-laden liposomes were mainly taken up by the galactose/fucose (Gal/Fuc)-recognizing receptors on liver macrophages (Kupffer cells) and not by the parenchymal ASGPr. More recently, it was demonstrated by Biessen et al.[23] that the binding of tris-gal-chol based clusters to the ASGPr could be improved dramatically by increasing the distance between the three galactose residues. Indeed, the most potent compound, TG-(20Å)C,[24] (**1**, Figure 1), effectively and selectively redirected high (HDL)- and low (LDL)-density lipoproteins[25] to the parenchymal liver cell. However, due to the relatively high hydrophilicity of the glycolipid, rapid exchange of glycolipid occurred, as judged from the moderate 20% liver uptake attained after preloading these lipid particles with TG(20Å)C. In addition, the chemical stability of TG(20Å) was rather poor, due to the acid-labile methylene acetal linkage in the 20 Å long spacer between each galactoside anomeric center and the branching point. The latter two phenomena imposed serious restrictions on the use of TG(20Å)C as a homing device for drug-containing liposomes.

In this article, we introduce a set of novel amphiphilic glycolipids **2**−**6** (Figure 2) in which, with respect to **1**, two important modifications are present. To enhance the stability of incorporation in the lipid bilayer of the liposome and to reduce untimely exchange to other lipoproteins or lipid compartments, the lipophilicity of the anchoring moiety was increased by adding one or more fatty acid chains to the steroid core structure. In addition, the methylene acetals connecting the glycol

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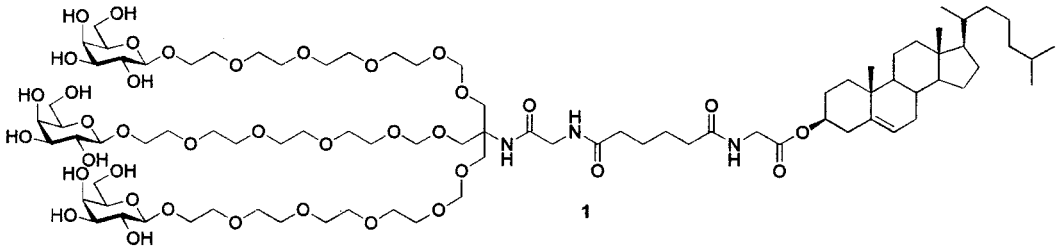


Figure 1. Chemical structure of TG(20Å)C.

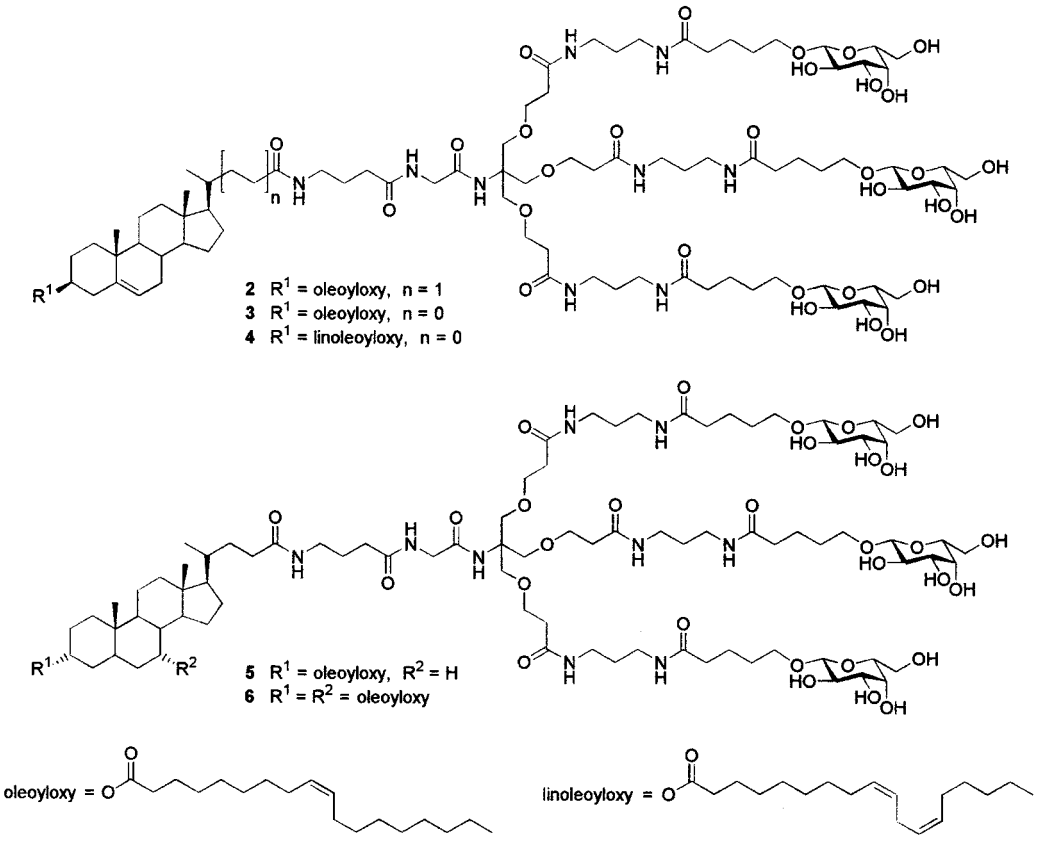


Figure 2. Chemical structures of the target glycolipids.

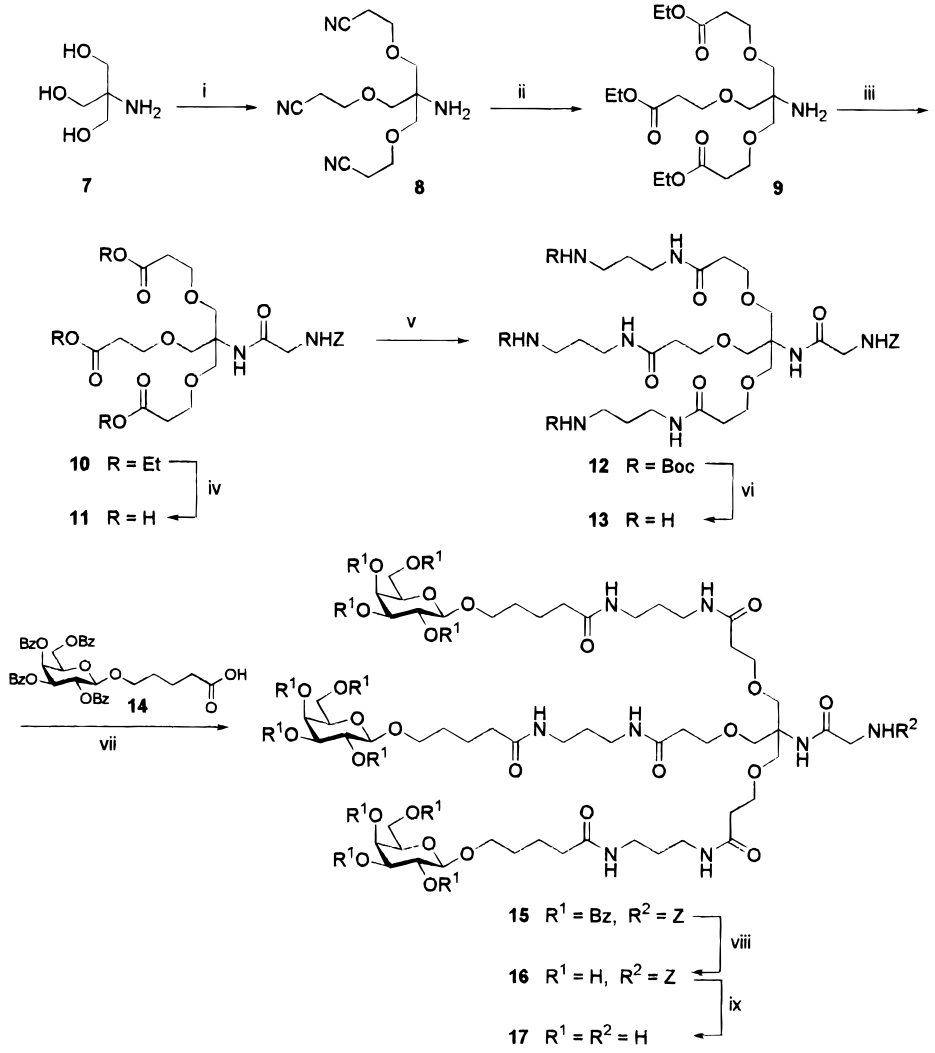
spacers to the TRIS cluster core in TG(20Å)C were replaced by more stable ether bonds. The glycolipids **2**−**6** were tested for their ability to associate with small unilamellar liposomes, their exchange behavior in serum, and their potential for targeting of these liposomes to the liver parenchymal cells in vivo.

Results and Discussion

Synthesis. Initially, attention was focused on the synthesis of the cluster galactoside moiety of the glycolipids. Very recently, a versatile galactoside building block (**14**, Scheme 1) equipped with a pentanoic acid spacer was introduced by Valentijn et al. for the solid-phase synthesis of peptide-based multivalent galactoside ligands.[9] Using this particular galactoside synthon as the terminus of each dendrimer arm, an 8-atom spacer moiety was required to establish a 20 Å long connection between each galactose and the branching point (Figure 3). On the basis of these prerequisites, the ether-bridged dendrimer moiety **B** was designed.

The synthesis of the ether-linked cluster galactoside **B** started with the alkylation of the three hydroxyl functions of TRIS (**7**) via a Michael addition to acrylonitril[26] (Scheme 1). The resulting aminotrinitrile **8** was then converted into the triethylester **9** by refluxing with a solution of hydrochloric acid in ethanol. Subsequently, the amino function of **9** was coupled with Z-Gly in high yield using DCC/HOBT as condensing reagent. Saponification of the ethyl esters of **10**, followed by HBTU/HOBT-mediated[27,28] condensation of the resulting trivalent carboxylic acid **11** with *N*-Boc-1,3-diaminopropane,[29] furnished the dendrimeric synthon **12**. The three aminopropyl groups of **12** were selectively deprotected with TFA in dichloromethane, leading to **13**. Condensation of the crude triamine **13** with the galactoside **14**, followed by selective removal of the benzoyl groups of **15** under Zemplen conditions, furnished the trivalent galactoside **16**. Finally, the *N*-benzyloxycarbonyl (Z) protective group on the glycinoyl-TRIS core unit was removed to allow functionalization of the cluster galac-

Scheme 1. Synthesis of the Cluster Galactoside Synthon 17[a]



[a] Reagents: (i) acrylonitril, KOH; (ii) HCl, EtOH; (iii) Z-Gly, DCC, HOBT; (iv) aq NaOH, dioxane; (v) $BocNH(CH_2)_3NH_2$, HBTU, HOBT; (vi) TFA; (vii) HBTU, HOBT; (viii) NaOMe; (ix) ammonium formate, 10% Pd/C.

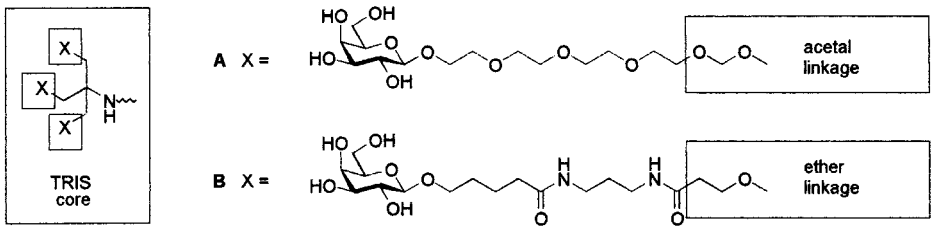


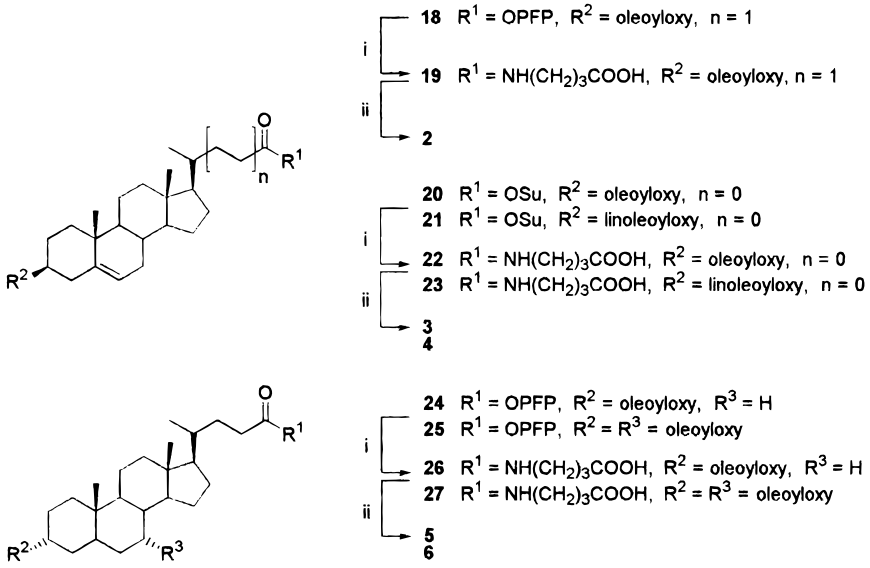
Figure 3. Structure of the spacer arms of the cluster galactoside moieties.

tosides with the lipophilic anchors. Deprotection of **16** proceeded smoothly with ammonium formate and Pd/C to give **17** in an excellent yield.

Having the key synthon **17** in hand, attention was now focused on the improvement of the lipophilic anchors of the target glycolipids. It has been reported[30−32] that the ability of synthetic cholesteryl esters of long chain fatty acids to associate with lipoproteins is substantially enhanced by the presence of one or more cis double bonds in these fatty acid side chains. These findings were confirmed recently in a study from our group[33] in which these lipophilic moieties were used for targeting of antisense oligonucleotides to the LDL receptor on tumor cells. On the basis of these results, the five bile acid ester moieties **18, 20, 21, 24**, and **25** were selected, all containing at least one unsaturated fatty acid side chain (Scheme 2). The bile acid esters differed in the structure of the steroid skeletons, the number of fatty acid tails, the orientation of the fatty acid tails, and the number of cis double bonds per fatty acid tail.

The bile acid esters were joined with the cluster galactoside via a γ-aminobutyric acid (GABA) linker. To this end (see Scheme 2), the pentafluorophenyl esters[33] **18**, **24**, and **25** and the *N*-succinimidyl esters[31] **20** and **21** were treated with GABA to afford the elongated

Scheme 2. Synthesis of the Glycolipids **2**−**6**[a]



[a] Reagents: (i) GABA; (ii) **17**, HBTU, HOBT.

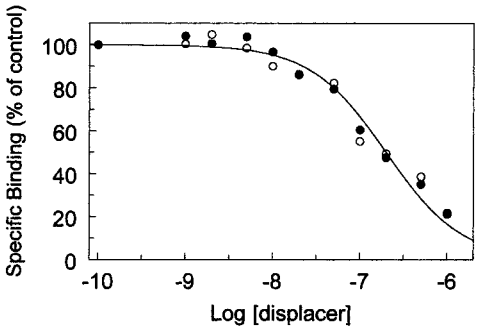


Figure 4. Competition curves of [^{125}I]ASOR binding to parenchymal cells for the cluster galactosides **16** and **28**. Parenchymal liver cells (1−1.5 × 10^6 cells/mL) were incubated for 2 h at 4 °C with ^{125}I-ASOR (5.5 nM) in the presence or absence of compound **16** (●) or compound **28** (○) at 10 concentrations, ranging from 1 nM to 1 μM. The specific binding of ^{125}I-ASOR, defined as the difference between total and nonspecific binding (determined in the presence of 100 mM GalNAc), is plotted as percentage of the control (without displacer) and versus the log of the concentration of displacer (M). Displacement binding data were analyzed according to a single site model using a computerized nonlinear fitting program (GraphPad).

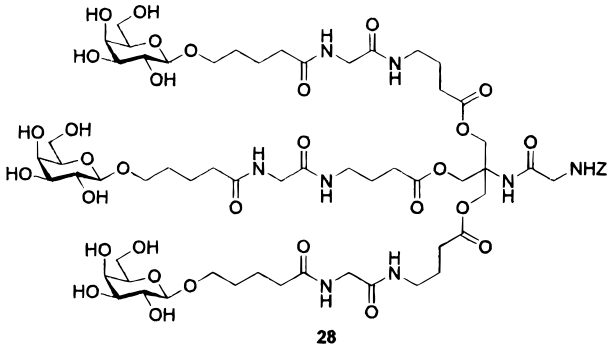


Figure 5. Structure of the ester-bridged cluster galactoside **28**.

derivatives **19**, **26**, **27**, **22**, and **23**, respectively. Finally, condensation with the glycinoyl-TRIS dendritic core **17** furnished the glycolipids **2**−**6**. Purification of **2**−**6** was performed by gel filtration over Sephadex LH20 in methanol. The homogeneity and identity of **2**−**6** was fully confirmed by NMR spectroscopy and mass spectrometry.

In Vitro Binding Studies. The affinity of compound **16** for the ASGPr was monitored by an in vitro competition assay of ^{125}I-ASOR binding to parenchymal rat liver cells. As can be seen from Figure 4, **16** displayed an affinity for the ASGPr (K_i = 93 nM, pK_i = 7.03 ± 0.04) that was similar to that of TG(20Å)C (K_i = 0.20 μM).[23] Moreover, in a separate study, the cluster galactoside **28**[34] (Figure 5), containing an equally long spacer moiety but having a different composition, also showed an affinity of 93 nM (pK_i = 7.03 ± 0.06). These data indicate that the affinity of a trivalent cluster galactoside for the ASGPr is governed less by the nature than by the length of the spacer arm, provided that maximum flexibility between the galactose residue and the branching point is maintained.

Association of Glycolipids with Liposomes. Using FPLC analysis, it appeared that all glycolipids form micelles with a size of 8−10 nm, which can easily be separated from the relatively large liposomes (27.0 ± 0.3 nm). Glycolipids readily incorporate into liposomes by virtue of their hydrophobic bile acid residues which are able to intercalate into the liposomal phospholipid bilayer. Indeed (see Figure 6), incubation of liposomes with increasing amounts of **5** (5, 10, and 50% w/w) resulted in a recovery of 100 ± 0%, 96.5 ± 0.5%, and 59.4 ± 1.0% (mean ± variation; n = 2) of the added glycolipid with the liposomes. Incorporation of glycolipid **5** did not substantially alter the liposomal size, as judged from the Sephacryl S-1000 elution profiles. Assuming the phospholipid content per liposome to be 1.31 × 10^{-17} g,[35] it can be calculated that at these incubation ratios approximately 190, 370, and 1140 molecules of glycolipid **5** do become associated per liposome, respectively. Similarly (see Figure 6), **2** and **3** both quantitatively incorporated in the liposomes at 5% (w/w). These results indicate that neither the

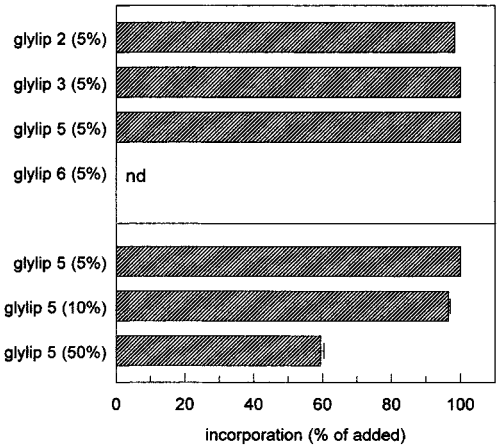


Figure 6. Association of glycolipids with liposomes. Liposomes (100 μg of phospholipids) were incubated with glycolipids **2**, **3**, and **6** (5%; w/w) or **5** (5, 10, and 50%; w/w) for 30 min at 37 °C. Liposomally incorporated glycolipid was separated from unincorporated glycolipid by FPLC, and the galactose content of the resulting fractions was determined. nd = not detectable.

orientation of the oleoyl tail, nor the structure of the steroid skeletons in glycolipids **2**, **3**, or **5** has any substantial effect on the level of incorporation. By contrast, association of **6** with the liposomes did not occur, indicating that the *number* of fatty acid tails does have a strong influence on incorporation. The presence of the two oleoyl tails in **6** may induce the formation of more stable glycolipid micelles than do the single oleoyl tails of the other glycolipids, and therefore the critical micellar concentration of **6** may be too low. The linoleoyl glycolipid **4** was found highly susceptible to oxidation in aqueous solutions and was therefore not used for further studies.

Liver Uptake and Serum Decay of Glycolipid/Liposome Complexes. Upon intravenous injection into mice, the liposomes showed a low uptake by the liver (7.7 ± 0.4% of the injected dose) and a high remaining fraction within the serum (81.3 ± 2.1%) at 30 min after injection, which is similar to that recently reported for rats (Figure 7).[35] Incubation of liposomes with increasing amounts of glycolipid **5** (0, 5, 10 and 50% w/w) resulted in a gradually decreased liposomal serum half-life from $t_{1/2} > 5$ h for the control liposomes to $t_{1/2} \approx 2-5$ min at 50% (w/w) **5** (see Figure 7). Concomitantly, the uptake of liposomes by the liver increased from 7.7% up to 83.5% of the injected dose at 30 min after injection (Figure 7). It is important to note that uptake of these liposomes by the spleen was negligible, indicating that the particles show a very low tendency to be eliminated by cells of the reticuloendothelial system (RES).

To discriminate between glycolipid-induced uptake of the liposomes by the ASGPr on liver parenchymal cells versus the Gal/Fuc-recognizing receptor on liver macrophages (Kupffer cells), we have tested the effect of asialofetuin (ASF), an established competitor of ASGPr-mediated uptake,[16,17,36] on the pharmacokinetics. Preinjection of ASF almost completely inhibited the liver uptake of liposomes enriched with 5% (w/w) **5** (see Figure 7). In contrast, the liver uptake of the 10% (w/w) loaded liposomes was only partially inhibited by ASF, and ASF even had no effect on the liver recognition of liposomes enriched with 50% (w/w) **5**. It can be calculated that with a loading of 5% (w/w) of **5**, the average liposomal surface area for each cluster galactoside is about 15 nm^2. With a loading of 50% (w/w), this area is reduced to about 3 nm^2. In the latter case, the conformational properties of the individual galactose clusters which are of vital importance for recognition by the ASGPr may be overruled by the high overall galactose density on the liposomal surface. It is well-known that high densities of galactosides at the liposome surface lead to efficient uptake by the Gal/Fuc-recognizing receptor located on liver macrophages (Kupffer cells).[37] It was therefore decided to establish the ability of the other glycolipids to direct liposomes to the parenchymal liver cells at a 5% (w/w) glycolipid content. The glycolipids **2** and **3** showed uptake-inducing characteristics that were similar to those found for glycolipid **5** (see Figure 8), including negligible uptake by the spleen. The dioleate glycolipid **6**, which was already shown not to incorporate into liposomes, displayed no effect on liposome uptake (Figure 8).

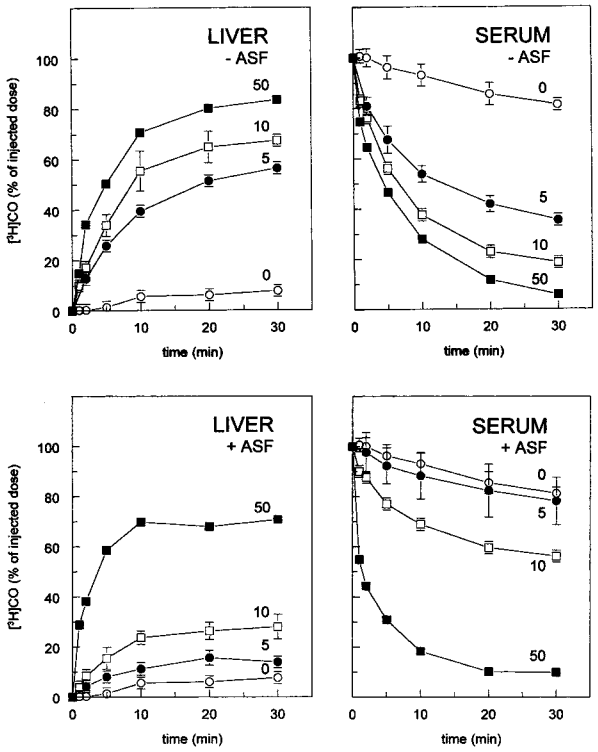


Figure 7. Liver uptake and serum decay of control and glycolipid **5**-laden liposomes in mice. [^{3}H]Cholesteryl oleate-labeled liposomes were injected into anesthetized mice, without (○) or with previous incubation with 5 (●), 10 (□), or 50 μg (■) of **5**. At the indicated times, the liver uptake (left) and serum decay (right) were determined, without (top) or with (bottom) previous injection of ASF (50 mg/kg) at 1 min before injection of the liposomes. Values are means ± variation of two experiments.

An important common feature of the present glycolipids **2**, **3**, and **5** is that they induce a monophasic serum decay of the particles, whereas a biphasic serum decay of lipidic particles such as LDL and HDL was induced by TG(20Å)C (**1**). Only a small portion of the TG(20Å)C-laden lipoproteins was cleared in the α-phase (20−30%).[25] These data can be explained by a rapid exchange of TG(20Å)C from the injected particles. Evidently, the present glycolipids **2**, **3**, and **5** do not redistribute from the liposomes upon injection, which is a major advan-

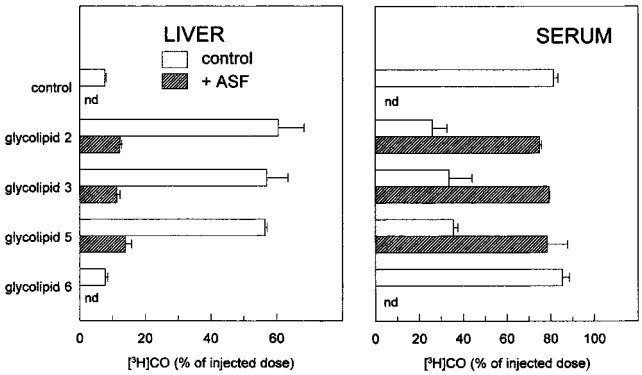


Figure 8. Liver and serum distribution of liposomes in mice. [^{3}H]Cholesteryl oleate-labeled control and glycolipid (5 μg)-laden liposomes (100 μg of phospholipid) were injected intravenously into anesthetized mice, without or with previous injection of ASF (50 mg/kg) at 1 min before injection of the liposomes. After 30 min of circulation, the uptake by the liver and the remaining fraction in the serum was determined (percentage of the injected dose). Liver values are corrected for serum radioactivity. Values are means ± variation of two experiments. nd = not determined.

tage as compared to TG(20Å)C when aiming at application as a homing device in parenchymal cell-specific targeting of liposomes.

In conclusion, we have prepared three glycolipids **2**, **3**, and **5** which are capable of associating with liposomes. At a loading ratio of 5% (w/w), the glycolipid/liposome particles are efficiently processed by the hepatic asialoglycoprotein receptor. The glycolipids presented here are in two aspects superior to the latest glycolipid introduced by our group for targeting to the ASGPr, TG-(20Å)C (**1**). First, the trivalent cluster galactoside moiety present in the glycolipids **2**, **3**, and **5** is chemically more accessible and stable, while the high affinity for the ASGPr is maintained. Second, no undesired exchange of the glycolipids **2**, **3**, and **5** from the liposomes to other lipid compartments (endogenous lipoproteins or cell membranes) could be detected. These advantageous properties open the way for the employment of glycolipids **2**, **3**, and **5** for the efficient targeting of amphiphilic or water-soluble drugs, such as antisense oligonucleotides, to the parenchymal liver cells.

Experimental Section

General. Pyridine, *N*,*N*-dimethylformamide (DMF) and 1,2-dichloroethane (DCE) were distilled from CaH_2 and subsequently stored over molecular sieves (4 Å). *N*,*N*-Diisopropylethylamine (DIPEA) was distilled from 4-toluenesulfonyl chloride and redistilled from KOH pellets. [1α,2α-^{3}H]Cholesteryl oleate ([^{3}H]CO) was from Amersham International (Little Chalfont, U.K.). Triolein (99% pure) and egg yolk phosphatidylcholine (EYPC) (98%) were from Fluka (Buchs, Switzerland). L-α-Lysophosphatidylcholine (99%) and cholesterol (>99%) were from Sigma Chemical Co. (St. Louis, MO). Cholesteryl oleate (CO) (97%) was from Janssen (Beersse, Belgium). Cholesterol oxidase, cholesterol esterase, peroxidase type II (200 U/mg), and Precipath L were from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade. Merck Kieselgel 60 F_{254} DC Alufolien was used for TLC analysis. Carbohydrate compounds were visualized by charring with sulfuric acid/ethanol (1/4, v/v), bile acid esters by charring with $MnCl_2$.[38] Compounds containing NH functions were visualized by charring with a solution of TDM[39] after treatment of the TLC plates with chlorine or visualized by charring with a solution of ninhydrine in acetic acid/water. Column chromatography was performed with Kieselgel 60, 230−400 mesh (Merck). Gel filtration was performed with Sephadex LH-20 (Pharmacia). ^{1}H NMR spectra (200 MHz) and ^{13}C{^{1}H} NMR spectra (50.1 MHz) were recorded using a JEOL JNM-FX200 spectrometer. ^{1}H NMR spectra (300 MHz) were recorded using a Bruker WM-300 spectrometer. 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. Ion spray mass spectrometry was carried out using a Perkin-Elmer API 165 mass spectrometer.

Syntheses. Tris(cyanoethoxymethyl)aminomethane (8). Tris(hydroxymethyl)aminomethane (**7**) (24.2 g; 0.2 mol) was added to a mixture of 1,4-dioxane (25 mL) and aqueous KOH (40%; 1.2 g). To this solution was added dropwise, under vigorous stirring, acrylonitril (40 mL; 0.61 mol). After 3 h, the temperature of the reaction mixture suddenly increased, and a slightly yellow color appeared. The mixture was cooled with an ice bath and left stirring overnight. The mixture was neutralized by the addition of aqueous HCl (2.5 M; 3.8 mL) and filtered. The filtrate was concentrated under reduced pressure. The resulting oil was dissolved in DCE and dried ($MgSO_4$). After filtration and concentration of the filtrate under reduced pressure, the crude product thus obtained was purified portionwise over a silica gel column, using DCM/methanol (1/0 → 88/12, v/v) as eluent.

^{13}C{^{1}H} NMR ($CDCl_3$): δ 117.6 (CN), 71.9 (CH_2 TRIS), 65.1 (OCH_2CH_2CN), 55.4 (C_q TRIS), 18.1 (CH_2CN).

Tris(carboxyethoxyethyl)aminomethane Triethyl Ester (9). The trinitrile **8** (1.4 g; 5.0 mmol) was added to a saturated solution of gaseous hydrogen chloride in dry ethanol (25 mL). The mixture was refluxed for 3 h and subsequently left stirring overnight at room temperature. Next, the mixture was filtered, and the filtrate was concentrated under reduced pressure. Water (40 mL) was added, and the mixture was neutralized with aqueous ammonia (25%). The mixture was extracted with DCM (2 × 50 mL), and the combined organic layers were dried over $MgSO_4$. The crude product was purified over a silica gel column using toluene/ethanol (1/0 → 94/6, v/v) as eluent.

Yield: 1.2 g (2.8 mmol, 56%). ^{13}C{^{1}H} NMR ($CDCl_3$): δ 171.2 (C=O), 72.5 (CH_2 TRIS), 66.6 (OCH_2CH_2), 60.1 (OCH_2CH_3), 55.7 (C_q TRIS), 34.8 (CH_2C=O), 14.0 (CH_3). ^{1}H NMR ($CDCl_3$): δ 4.15 (q, 6 H, OCH_2CH_3, J = 7.1 Hz), 3.69 (t, 6 H, OCH_2CH_2, J = 6.3 Hz), 3.32 (s, 6 H, CH_2 TRIS), 2.55 (t, 6 H, CH_2C=O), 1.27 (t, 9 H, CH_3).

***N*-(*N*-Benzyloxycarbonyl-glycinyl)-tris(carboxyethoxymethyl)aminomethane Triethyl Ester (10).** To a solution of *N*-benzyloxycarbonyl-glycine (Z-Gly) (1.3 g; 6.2 mmol), **9** (2.6 g; 6.2 mmol), and HOBT (0.84 g; 6.2 mmol) in acetonitrile (80 mL) was added DCC (1.3 g; 6.2 mmol). After the mixture had been left stirring overnight, TLC analysis (DCM/methanol 9/1, v/v) revealed complete conversion of the starting materials into one product. The mixture was filtered, concentrated, taken up in DCM (200 mL), washed with dilute H_3PO_4 (1 M; 100 mL), aqueous $NaHCO_3$ (1 M; 100 mL), and brine (100 mL), dried ($MgSO_4$), and evaporated to a small volume. The resulting oil was applied to a silica gel column using DCM/methanol (1/0 → 96/4, v/v) as eluent.

Yield: 3.5 g (5.7 mmol, 92%). ^{13}C{^{1}H} NMR ($CDCl_3$): δ 171.0 (C=O ester), 168.5 (C=O Gly), 155.9 (C=O Z), 136.0 (C_q Z), 127.8, 127.4 (CH_{arom} Z), 68.4 (CH_2 TRIS), 66.2 (CH_2 Z, OCH_2CH_2), 59.8 (OCH_2CH_3), 59.3 (C_q TRIS), 44.1 (CH_2 Gly), 34.3 (CH_2C=O), 13.6 (CH_3). ^{1}H NMR ($CDCl_3$): δ 7.37−7.32 (m, 5 H, CH_{arom} Z), 5.12 (s, 2 H, CH_2 Z), 4.13 (q, 6 H, CH_2CH_3, J = 7.2 Hz), 3.86 (d, 2 H, CH_2 Gly, J = 5.5 Hz), 3.69 (s, 6 H, CH_2 TRIS), 3.67 (t, 6 H, OCH_2CH_2, J = 6.2 Hz), 2.52 (t, 6 H, CH_2C=O), 1.25 (t, 9 H, CH_3).

***N*-(*N*-Benzyloxycarbonyl-glycinyl)-tris-(carboxyethoxymethyl)aminomethane, $[HOC(O)(CH_2)_2OCH_2]_3$-$CNHC(O)CH_2NHZ$ (11).** The triester **10** (0.31 g; 0.50 mmol) was dissolved in a mixture of 1,4-dioxane (30 mL) and water (9 mL). To this solution was added aqueous NaOH (4 M; 1 mL). After stirring for 3 h, TLC analyis (DCM/methanol/acetic acid 18/2/1, v/v/v) showed complete conversion of the starting

material into one product. The mixture was acidified by the addition of aqueous HCl (36−38%; 1 mL). Water (50 mL) was added, and the resulting mixture was extracted with DCM (3 × 50 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to an oil. The crude **11** thus obtained was immediately used for the synthesis of **12**.

$^{13}C\{^1H\}$ NMR (CD_3OD): δ 175.4 (*C*=O carboxyl), 171.7 (*C*=O amide), 129.4−128.8 (CH_{arom} Z), 69.9 (CH_2 TRIS), 68.0 (OCH_2CH_2), 67.8 (CH_2 Z), 61.2 (C_q TRIS), 45.1 (CH_2 Gly), 35.6 (CH_2C=O).

[BocNH(CH$_2$)$_3$NHC(O)(CH$_2$)$_2$OCH$_2$]$_3$CNHC(O)CH$_2$NHZ (12). Crude **11** (0.50 mmol), *N*-*tert*-butoxycarbonyl-1,3-diaminopropane[29] (BocDAP), (0.52 g; 3.0 mmol), DIPEA (0.91 mL; 5.3 mmol) and HOBT (0.24 g; 1.8 mmol) were dissolved in DMF (20 mL). To this solution was added HBTU (0.68 g; 1.8 mmol). The reaction was left stirring overnight at 4 °C. According to TLC analysis (DCM/methanol 9/1, v/v), the reaction was complete. The mixture was taken up in DCM (100 mL), washed with dilute H_3PO_4 (1 M; 100 mL), aqueous $NaHCO_3$ (1 M; 100 mL), and brine (100 mL), dried ($MgSO_4$), concentrated, and coevaporated with toluene (2 × 20 mL) and ethanol (20 mL). The resulting oil was applied to a silica gel column, using DCM/methanol (1/0 → 92/8, v/v) as eluent. Crude **12** thus obtained was further purified by Sephadex LH-20 gel filtration, using DCM/methanol (2/1, v/v) as eluent.

Yield: 0.38 g (0.38 mmol, 76% based on **10**). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 171.8 (*C*=O amide), 169.5 (*C*=O Gly), 156.7, 156.3 (*C*=O Z, Boc), 136.2 (C_q Z), 128.5−127.6 (CH_{arom} Z), 78.9 (C_q Boc), 69.3 (CH_2 TRIS), 67.3 (OCH_2CH_2), 66.6 (CH_2 Z), 59.7 (C_q TRIS), 44.7 (CH_2 Gly), 37.3, 36.3, 29.6 (CH_2 DAP), 36.3 (CH_2C=O), 28.2 (CH_3 Boc). ^1H NMR ($CDCl_3$): δ 7.35−7.27 (m, 5 H, CH_{arom} Z), 5.12 (s, 2 H, CH_2 Z), 3.85 (d, 2 H, CH_2 Gly, J = 5.1 Hz), 3.64 (broad, 12 H, CH_2 TRIS, OCH_2CH_2), 3.28 (q, 6 H, CH_2N DAP, J = 6.0 Hz), 3.13 (t, 6 H, CH_2N DAP, J = 6.0 Hz), 2.40 (t, 6 H, CH_2C=O, J = 6.5 Hz), 1.63 (m, 6 H, CH_2 DAP), 1.43 (s, 27 H, CH_3 Boc). Mass (ion spray +): *m*/*e* 1020 [M + Na^+].

[Bz$_4$GalO(CH$_2$)$_4$C(O)NH(CH$_2$)$_3$NHC(O)(CH$_2$)$_2$OCH$_2$]$_3$CNHC(O)CH$_2$NHZ (15). Compound **12** (0.25 g; 0.25 mmol) was dissolved in TFA/DCM (1/4, v/v; 2.5 mL). After the mixture was stirred for 30 min, toluene (15 mL) was added. The mixture was concentrated, and coevaporated with toluene (3 × 10 mL). According to TLC analysis (methanol/DCM/25% NH_4OH 6/14/2, v/v/v), the starting material was completely converted into one product.

The crude triamine **13** thus obtained was dissolved in DMF (10 mL), and 5-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyloxy)pentanoic acid (**14**) (0.58 g; 0.83 mmol), DIPEA (0.37 mL; 2.1 mmol), and HOBT (0.12 g; 0.90 mmol) were added. To this mixture was added HBTU (0.34 g; 0.9 mmol). The reaction was left stirring overnight at 4 °C. According to TLC analysis (DCM/methanol 9/1, v/v), the reaction was complete. The mixture was concentrated, and coevaporated with toluene (2 × 20 mL). The mixture was taken up in DCM (50 mL), washed with dilute H_3PO_4 (1 M; 50 mL), aqueous $NaHCO_3$ (1 M; 50 mL), and water (50 mL), dried ($MgSO_4$), concentrated, and coevaporated with toluene (2 × 20 mL) and ethanol (20 mL). The crude product was purified by silica gel column chromatography, using DCM/methanol (1/0 → 92/8, v/v) as eluent, followed by Sephadex LH-20 gel filtration, using DCM/methanol (2/1, v/v) as eluent.

Yield: 0.56 g (0.21 mmol, 82% based on **12**). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 173.3, 171.7 (2 × *C*=O amide), 169.2 (*C*=O Gly), 165.9, 165.3 (*C*=O benzoyl), 156.6 (*C*=O Z), 136.3 (C_q Z), 133.4−127.6 (CH_{arom}), 129.2, 129.1, 128.9 (C_q benzoyl), 101.4 (C-1 Gal), 71.5, 71.1, 69.8, 68.0 (C-2,3,4,5 Gal), 69.9, 69.4 (CH_2 TRIS, OCH_2 pentanoyl), 67.4 (OCH_2 propanoyl), 66.6 (CH_2 Z), 61.8 (C-6 Gal), 59.7 (C_q TRIS), 44.6 (CH_2 Gly), 36.6, 35.9, 35.7 (CH_2N DAP, CH_2C=O propanoyl, CH_2C=O pentanoyl), 29.4, 28.5 (CH_2 DAP, CH_2 pentanoyl), 22.1 (CH_2 pentanoyl). ^1H NMR ($CDCl_3$): δ 8.10−7.13 (m, 25H, CH_{arom} benzoyl, Z), 6.02 (d, 3 H, H-4 Gal, J_{3-4} = 3.1 Hz), 5.80 (dd, 3 H, H-2 Gal, J_{1-2} = 7.6 Hz), 5.66 (dd, 3 H, H-3 Gal, J_{2-3} = 9.5 Hz), 5.11 (s, 2 H, CH_2 Z), 4.86 (d, 3 H, H-1 Gal), 4.75−4.36 (m, 9 H, H-5, H-6a,b Gal), 4.04−3.57 (m, 20 H, CH_2 Gly, OCH_2 propanoyl, CH_2 TRIS, OCH_2 pentanoyl), 3.23−3.19 (m, 12 H, 2 × CH_2N DAP), 2.40−2.36 (m, 6 H, CH_2C=O propanoyl), 2.10−2.04 (m, 6 H, CH_2C=O pentanoyl), 1.60 (broad, 18 H, CH_2 propanoyl/pentanoyl).

[GalO(CH$_2$)$_4$C(O)NH(CH$_2$)$_3$NHC(O)(CH$_2$)$_2$OCH$_2$]$_3$CNHC(O)CH$_2$NHZ, ZGly-TRISGal$_3$ (16). To a solution of compound **15** (0.56 g; 0.21 mmol) in dry methanol (50 mL) was added a solution of NaOMe in methanol (30%, 1 mL). The mixture was stirred for 48 h at 4 °C, until TLC analysis (*n*-propanol/*n*-butanol/water/25% NH_4OH 40/15/15/30, v/v/v/v) revealed the reaction to be complete. The mixture was neutralized by the addition of Dowex (50 W × 8, H^+ form), filtered, and concentrated to a small volume. The crude product thus obtained was purified by Sephadex LH-20 gel filtration, using methanol as eluent.

Yield: 0.28 g (0.19 mmol, 91%). $^{13}C\{^1H\}$ NMR (D_2O): δ 177.2, 174.7 (2 × *C*=O amide), 172.1 (*C*=O Gly), 159.0 (*C*=O Z), 137.1 (C_q Z), 129.5−128.4 (CH_{arom} Z), 103.6 (C-1 Gal), 75.8 (C-5 Gal), 73.6 (C-3 Gal), 71.5 (C-2 Gal), 70.5 (OCH_2 pentanoyl), 69.4 (C-4 Gal, CH_2 TRIS), 68.3 (OCH_2 propanoyl), 67.9 (CH_2 Z), 61.7 (C-6 Gal), 60.8 (C_q TRIS), 44.8 (CH_2 Gly), 37.6, 37.4 (2 × CH_2N DAP), 37.0 (CH_2C=O propanoyl), 36.2 (CH_2C=O pentanoyl), 29.0, 28.8 (CH_2 DAP, 2 × CH_2 pentanoyl), 22.7 (CH_2 pentanoyl). ^1H NMR (D_2O): δ 7.43−7.38 (m, 5 H, CH_{arom} Z), 5.13 (s, 2 H, CH_2 Z), 4.36 (d, 3 H, H-1 Gal, J_{1-2} = 7.8 Hz), 3.90 (d, 3 H, H-4 Gal, J_{3-4} = 3.5 Hz), 3.95−3.57 (m, 29 H, CH_2 Gly, OCH_2 propanoyl, H-5 Gal, H-6a,b Gal, CH_2 TRIS, OCH_2 pentanoyl), 3.63 (dd, 3 H, H-3 Gal, J_{2-3} = 9.9 Hz), 3.50 (dd, 3 H, H-2 Gal), 3.19 (m, 12 H, 2 × CH_2N DAP), 2.44 (t, 6 H, J = 5.6 Hz, CH_2C=O propanoyl), 2.25 (t, 6 H, J = 6.9 Hz, CH_2C=O pentanoyl), 1.73−1.60 (m, 18 H, CH_2 DAP, 2 × CH_2 pentanoyl). Mass (FAB^-): *m*/*e* 1482.

[GalO(CH$_2$)$_4$C(O)NH(CH$_2$)$_3$NHC(O)(CH$_2$)$_2$OCH$_2$]$_3$CNHC(O)CH$_2$NH$_2$, Gly-TRISGal$_3$ (17). To a solution of **16** (99 mg; 67 μmol) in methanol (2 mL) was added Pd/C (10%; 50 mg) and ammonium formate (100 mg). The mixture was gently shaken for 2 h. According to TLC analysis (*n*-propanol/*n*-butanol/water/25% NH_4OH 40/15/15/30, v/v/v/v), the reaction was complete. The mixture was filtered over a path of Celite, concentrated, coevaporated with water/methanol (1/1, v/v; 3 × 20 mL), and lyophilized from water.

Yield: 90 mg (67 μmol, >99%). $^{13}C\{^1H\}$ NMR (D_2O): δ 177.3, 174.6 (2 × *C*=O amide), 103.5 (C-1 Gal), 75.8, 73.5, 71.4, 69.3 (C-2,3,4,5 Gal), 70.4 (OCH_2 pentanoyl), 69.1 (CH_2 TRIS), 68.2 (OCH_2 propanoyl), 61.6 (C-6 Gal), 61.2 (C_q TRIS), 37.5, 37.4, 36.9, 36.1 (CH_2N DAP, CH_2C=O propanoyl, CH_2C=O pentanoyl), 28.8, 28.7 (CH_2 DAP, CH_2 pentanoyl), 22.6 (CH_2 pentanoyl). Mass (FAB^-): *m*/*e* 1348.

***N*-(3β-(Oleoyloxy)-5-cholenoyl)-4-aminobutyric Acid, ((Oleoyloxy)-5-cholenoyl)-GABA (19).** To a solution of 3β-(oleoyloxy)-5-cholenic acid pentafluorophenyl ester (**18**) (53 mg; 66 μmol) in DCE/DMF (1/1, v/v; 2 mL) was added triethylamine (21 μL; 150 μmol) and 4-aminobutyric acid (10 mg; 75 μmol). The mixture was stirred overnight at room temperature, until TLC analysis (DCM/MeOH 9/1, v/v) indicated complete conversion of starting material into a product with zero mobility. The mixture was concentrated under reduced pressure, coevaporated with toluene (2 × 10 mL) and purified by silica gel column chromatography, using DCM/MeOH (1/0 → 84/6, v/v) as eluent.

Yield: 32 mg (44 μmol, 67%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 174.7 (*C*=O GABA), 173.1 (*C*=O ester, C-24), 139.8 (C-5), 130.0, 129.7 (2 × *C*H oleoyl), 122.4 (C-6), 73.4 (C-3), 56.7, 55.9 (C-14, C-17), 50.0 (C-9), 35.7 (C-20), 19.3 (C-19), 18.5 (C-21), 14.1 (CH_3 oleoyl), 11.9 (C-18). Mass (ion spray +): *m*/*e* 747 [M + Na^+].

***N*-(3β-(Oleoyloxy)-bisnor-5-cholenoyl)-4-aminobutyric Acid, ((Oleoyloxy)-bisnor-5-cholenoyl)-GABA (22).** To a solution of 3β-(oleoyloxy)-bisnor-5-cholenic acid *N*-hydroxysuccinimidyl ester (**20**) (171 mg; 242 μmol) in freshly distilled THF (2 mL) were added triethylamine (73 μL; 1.0 μmol) and 4-aminobutyric acid (50 mg; 0.48 mmol). The mixture was stirred for 48 h at room temperature and subsequently 48 h

at 40 °C. According to TLC analysis (DCM/MeOH 9/1, v/v), **20** was converted completely into baseline material. Workup and purification as described for **19** afforded pure **22**.

Yield: 75 mg (108 μmol, 45%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 177.6 (*C*═O GABA), 173.2 (C-22, *C*═O ester), 139.7 (C-5), 130.0, 129.7 (2 × *C*H oleoyl), 122.3 (C-6), 73.6 (C-3), 56.2 (C-17), 52.3 (C-14), 49.9 (C-9), 44.6 (C-20), 19.3 (C-19), 17.6 (C-21), 14.0 (*C*H_3 oleoyl), 12.0 (C-18). Mass (ion spray +): *m/e* 719 [M + Na^+].

***N*-(3β-(Linoleoyloxy)-bisnor-5-cholenoyl)-4-aminobutyric Acid, ((Linoleoyloxy)-bisnor-5-cholenoyl)-GABA (23).** Compound **23** was prepared from 3β-(linoleoyloxy)-bisnor-5-cholenic acid hydroxysuccinimidyl ester (**21**) (161 mg; 228 μmol) and 4-aminobutyric acid (47 mg; 0.46 mmol) as described for the synthesis of **22**.

Yield: 75 mg (108 μmol, 47%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 177.5 (*C*═O GABA), 173.1 (C-22, *C*═O ester), 139.8 (C-5), 130.2, 130.0, 128.0, 127.9 (4 × *C*H linoleoyl), 122.3 (C-6), 73.6 (C-3), 56.3 (C-17), 52.7 (C-14), 50.0 (C-9), 44.2 (C-20), 31.9 (C-8), 19.3 (C-19), 17.7 (C-21), 14.0 (*C*H_3 linoleoyl), 12.2 (C-18). Mass (ion spray +): *m/e* 717 [M + Na^+].

***N*-(3α-(Oleoyloxy)-5β-cholanoyl)-4-aminobutyric Acid, ((Oleoyloxy)-5β-cholanoyl)-GABA (26).** Compound **26** was prepared from 3α-(oleoyloxy)-5β-cholanic acid pentafluorophenyl ester (**24**) (102 mg; 126 μmol) and 4-aminobutyric acid (16 mg; 0.15 mmol) as described for the synthesis of **19**.

Yield: 49 mg (67 μmol, 54%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 175.1 (*C*═O GABA), 173.3 (*C*═O ester, C-24), 130.0, 129.7 (2 × *C*H oleoyl), 74.0 (C-3), 56.5, 56.2 (C-14, C-17), 42.0, 40.5 (C-5, C-9), 35.9, 35.6 (C8, C20), 23.4 (C-19), 18.5 (C-21), 14.1 (*C*H_3 oleoyl), 12.1 (C-18). Mass (ion spray +): *m/e* 749 [M + Na^+].

***N*-(3α,7α-Bis(oleoyloxy)-5β-cholanoyl)-4-aminobutyric Acid, ((Bis(oleoyloxy)-5β-cholanoyl)-GABA (27).** Compound **27** was prepared from 3α,7α-bis(oleoyloxy)-5β-cholanic acid pentafluorophenyl ester (**25**) (175 mg; 161 μmol) and 4-aminobutyric acid (20 mg; 0.20 mmol) as described for the synthesis of **19**.

Yield: 60 mg (60 μmol, 37%). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 176.3 (*C*═O GABA), 175.0 (C-24), 173.3, 173.1 (2 × *C*═O ester), 130.0, 129.7 (*C*H oleoyl), 73.8 (C-3), 71.0 (C-7), 55.9 (C-17), 50.4 (C-14), 41.0 (C-5), 38.0 (C-8), 35.5 (C-20), 34.1 (C-9), 22.7 (C-19), 18.4 (C-21), 14.0 (2 × *C*H_3 oleoyl), 11.7 (C-18). Mass (ion spray +): *m/e* 1029 [M + Na^+].

((Oleoyloxy)-5-cholenoyl)-GABA-Gly-TRISGal$_3$ (2). Compound **19** (24 mg; 33 μmol) was dissolved in DMF/DCE (1/1, v/v; 2 mL), and DIPEA (11 μL; 83 μmol), HOBT (6 mg; 40 μmol), and HBTU (16 mg; 40 μmol) were added. After the mixture was stirred for 15 min at room temperature, a solution of **17** (40 mg; 30 μmol) was added. The mixture was stirred overnight until, according to TLC analysis (2-propanol/water 4/1, v/v), the reaction was complete. The mixture was concentrated under reduced pressure, and coevaporated with toluene (3 × 10 mL) and ethanol (10 mL). The resulting oil was purified by silica gel column chromatography, applying 2-propanol/water (4/1, v/v) as eluent. Crude **2** thus obtained was further purified by Sephadex LH-20 gel filtration, using methanol as eluent.

Yield: 17 mg (8 μmol; 24%). $^{13}C\{^1H\}$ NMR (CD_3OD): δ 176.2−171.36 (*C*═O), 141.0 (C-5), 130.9, 130.7 (*C*H oleoyl), 123.6 (C-6), 105.0 (C-1 Gal), 76.6, 75.0, 72.6, 70.3 (C-2,3,4,5 Gal), 75.3 (C-3), 70.2, 68.7 (O*C*H_2 TRIS, pentanoyl, propanoyl), 62.5 (C-6 Gal), 61.5 (*C*$_q$ TRIS), 58.1, 57,2 (C-14, C-17), 51.6 (C-9), 33.2 (C-8, C-20), 19.7 (C-19), 18.9 (C-21), 14.4 (*C*H_3 oleoyl), 12.3 (C-18). Mass (FAB^+): *m/e* 2078 [M + $Na]^+$.

((Oleoyloxy)-bisnor-5-cholenoyl)-GABA-Gly-TRISGal$_3$ (3). Compound **3** was prepared from **22** (41 mg; 59 μmol) and **17** (59 mg; 44 μmol) as described for the synthesis of **2**.

Yield: 28 mg (14 μmol, 31%). $^{13}C\{^1H\}$ NMR (CD_3OD): δ 179.6−171.3 (*C*═O), 141.0 (C-5), 130.9, 130.7 (*C*H oleoyl), 123.5 (C-6), 105.0 (C-1 Gal), 76.6, 75.0, 72.6, 70.3 (C-2,3,4,5 Gal), 75.2 (C-3), 70.2, 68.7 (O*C*H_2 TRIS, pentanoyl, propanoyl), 62.5 (C-6 Gal), 61.5 (*C*$_q$ TRIS), 57.7 (C-17), 54.0 (C-14), 51.6 (C-9), 45.0 (C-20), 33.2 (C-8), 19.8 (C-9), 18.0 (C-21), 14.4 (*C*H_3 oleoyl), 12.5 (C-18). Mass (FAB^+): *m/e* 2049 [M + $Na]^+$.

((Linoleoyloxy)-bisnor-5-cholenoyl)-GABA-Gly-TRISGal$_3$ (4). Compound **4** was prepared from **23** (38 mg; 55 μmol) and **17** (59 mg; 44 μmol) as described for the synthesis of **2**.

Yield: 29 mg (14 μmol, 32%). $^{13}C\{^1H\}$ NMR (CD_3OD): δ 179.6−171.3 (*C*═O), 141.0 (C-5), 130.8, 129.2, 129.1, 129.0 (*C*H linoleoyl), 123.6 (C-6), 105.0 (C-1 Gal), 76.6, 75.0, 72.6, 70.3 (C-2,3,4,5 Gal), 75.2 (C-3), 70.2, 68.7 (O*C*H_2 TRIS, pentanoyl, propanoyl), 62.5 (C-6 Gal), 61.5 (*C*$_q$ TRIS), 57.8 (C-17), 54.0 (C-14), 51.5 (C-9), 45.0 (C-20), 33.2 (C-8), 19.8 (C-9), 18.0 (C-21), 14.4 (*C*H_3 linoleoyl), 12.5 (C-18). Mass (FAB^+): *m/e* 2048 [M + $Na]^+$.

((Oleoyloxy)-5β-cholanoyl)-GABA-Gly-TRISGal$_3$ (5). Compound **5** was prepared from **26** (34 mg; 46 μmol) and **17** (59 mg; 44 μmol) as described for the synthesis of **2**.

Yield: 25 mg (12 μmol, 28%). $^{13}C\{^1H\}$ NMR (CD_3OD): δ 176.7−171.4 (*C*═O), 130.9, 130.8 (*C*H oleoyl), 105.0 (C-1 Gal), 76.6, 75.1, 72.6, 70.4 (C-2,3,4,5 Gal), 75.6 (C-3), 70.2, 68.4 (O*C*H_2 TRIS, pentanoyl, propanoyl), 62.5 (C-6 Gal), 61.5 (*C*$_q$ TRIS), 57.8, 57.5 (C-14, C-17), 43.3, 41.8 (C-5, C-9), 37.2, 36.9 (C-8, C-20), 18.9 (C-21), 14.4 (*C*H_3 oleoyl), 12.5 (C-18). Mass (FAB^+): *m/e* 2079 [M + $Na]^+$.

((Bis(oleoyloxy)-5β-cholanoyl)-GABA-Gly-TRISGal$_3$ (6). Compound **6** was prepared from **27** (21 mg; 20 μmol) and **17** (32 mg; 24 μmol) as described for the synthesis of **2**.

Yield: 9 mg (4 mmol, 20%). $^{13}C\{^1H\}$ NMR (CD_3OD): δ 176.2−174.0 (*C*═O), 131.0, 130.8, 130.7 (*C*H oleoyl), 105.0 (C-1 Gal), 76.6, 75.0, 72.6, 70.4 (C-2,3,4,5 Gal), 75.4 (C-3), 72.7 (C-7), 70.2, 68.7 (O*C*H_2 TRIS, pentanoyl, propanoyl), 62.5 (C-6 Gal), 61.5 (*C*$_q$ TRIS), 57.5 (C-17), 51.8 (C-14), 42.3 (C-5), 39.3 (C-8), 36.9 (C-20), 23.1 (C-19), 18.9 (C-21), 14.5 (*C*H_3 oleoyl), 14.5 (*C*H_3 oleoyl), 10.2 (C-18). Mass (FAB^+): *m/e* 2358 [M + $Na]^+$.

In Vitro Binding Assay

Displacement of ^{125}I-ASOR binding to hepatocytes was determined as follows. Parenchymal liver cells (1−1.5 × 10^6 cells; viability > 90%) were incubated in 1 mL of Dulbecco's modified essential medium containing 2% BSA, with ^{125}I-ASOR (5.5 nM) in the presence or absence of unlabeled displacer at 10 concentrations, ranging from 1 nM to 1 μM. Following incubation for 2 h at 4 °C under gentle agitation, the medium was removed by aspiration and the cells were washed twice with 2 mL of ice-cold medium containing 0.2% BSA and once with medium lacking BSA. Subsequently, cells were counted for radioactivity. Cell binding was corrected for protein content. Nonspecific binding was measured in the presence of 100 mM GalNAc. Displacement binding data were analyzed according to a single-site model using a computerized nonlinear fitting program (GraphPad) to calculate the K_i.

Animals. Male 10−12-week-old C57Bl/6KH mice of mass 21−24 g from Broekman Instituut BV (Someren, The Netherlands), fed ad libitum with regular chow, were used for the in vivo experiments.

Preparation and Characterization of Liposomes. Liposomes were prepared by sonication as recently described.[35] In short, egg yolk phosphatidylcholine (25 mg), cholesteryl oleate (1 mg), and [^{3}H]cholesteryl oleate (100 μCi) were hydrated in 11.4 mL of 0.1 M KCl and 0.01 M Tris·HCl, pH 8.0, and subsequently sonicated for 1 h at 54 °C using a Soniprep 150 (MSE Scientific Instruments, Crawley, U.K.) at 18 μm output. The liposomes were purified and concentrated (1.014 g/mL) by density gradient ultracentrifugation according to Redgrave et al.[40] using NaCl/KBr/EDTA density solutions in a Beckman SW 40 Ti rotor at 40 000 rpm for 18−22 h at 4 °C. The liposomes were homogeneous with

respect to size (low polydispersity of 0.25−0.26), and the mean particle diameter was 27.0 ± 0.3 nm (mean ± variation of two independent experiments) as determined by photon correlation spectroscopy (Malvern 4700 C system; Malvern Instruments, Malvern, U.K.). Measurements were performed at 27 °C and a 90° angle between laser and detector. The phosphatidylcholine content was determined using the Boehringer Mannheim (Mannheim, Germany) enzymatic kit for phosphatidylcholine. Precipath L was used as an internal standard. The particles were stored at room temperature under argon and used for characterization and metabolic studies within 7 days following preparation.

Incorporation of Glycolipids into Liposomes. The freeze-dried glycolipids were dissolved in phosphate-buffered saline (PBS) at a final concentration of 50 mg/mL and stored at −80 °C under argon. Their stability was routinely checked by thin-layer chromatography (*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. The glycolipids (0−50 μg) were incubated with the liposomes (100 μg of phospholipids) for 30 min at 37 °C. The incorporation efficiency was determined by separating liposomally incorporated and free glycolipid by fast protein liquid chromatography (SMART system; Pharmacia Biotech AB, Uppsala, Sweden), using a Superose-6 or Sephacryl S-1000 column at a flow rate of 50 μL/min and with PBS (pH 7.4) as eluent. The galactose content of the resulting fractions was determined using a galactose oxidase assay (recovery 85−100%). The free glycolipids eluted similarly as HDL (8−10 nm), due to micelle formation. By contrast, in the presence of liposomes all glycolipids, except for glycolipid **6**, coeluted with the liposomes in the void volume, indicating efficient liposomal incorporation.

Liver Uptake and Serum Decay of (Glycolipid-Laden) Liposomes in Mice. Mice were anesthetized by subcutaneous injection of a mixture of ketamine (120 mg/kg body weight), Thalamonal (0.03 mg/kg fentanyl and 1.7 mg/kg droperidol), and Hypnorm (1.2 mg/kg fluanisone and 0.04 mg/kg fentanyl citrate), and the abdomens were opened. [^{3}H]Cholesteryl oleate-labeled liposomes (100 μg of phospholipid) were injected via the inferior vena cava, after previous incubation with PBS or glycolipids (5, 10, or 50 mg) for 30 min at 37 °C. When indicated, mice received a preinjection of enzymatically desialylated fetuin (asialofetuin, ASF) (50 mg/kg) 1 min before injection of the liposomes. At the indicated times, blood samples (<50 μL) and liver lobules were taken and processed as described in the literature.[41] At 30 min after injection, the mice were sacrificied, and their organs were excised and weighed. ^{3}H radioactivity in duplicate serum samples of 10 μL was counted in 2.5 mL of Emulsifier Safe (Packard, Downers Grove, IL). The total serum volumes of C57Bl/6KH mice were 1.068 ± 0.066 mL, as previously determined.[42] The tissue samples were solubilized in 0.5 mL of Soluene-350 (Packard) for approximately 5 h at 65 °C and bleached by adding 25 μL of 30% (v/v) H_2O_2. Subsequently, their radioactivity was counted in 15 mL of Hionic Fluor (Packard) and corrected for the serum radioactivity in the tissues at the time of sampling.[42]

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