Synthesis of Cluster Galactosides with High Affinity for the Hepatic Asialoglycoprotein Receptor#

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High-affinity ligands for the asialoglycoprotein receptor, which is uniquely localized on the parenchymal liver cell and recognizes oligoantennary galactosides, might be utilized as homing device to specifically target drugs or genes to parenchymal liver cells. In the present study, the synthesis of galactose-terminated triantennary glycosides, provided with various spacers between the β-galactopyranosyl moieties and the branching point of the dendrite, is described. *N*-[Tris[[(methylthio)methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipy)]glycinamide (**3b**) was glycosylated with monogalactosyl derivatives, containing propanediol or ethylene glycol units as hydrophilic spacer moieties, to yield the corresponding cluster galactosides. To determine the affinity of the cluster galactosides for the asialoglycoprotein receptor, we have performed competition studies of [^{125}I]ASOR binding, a specific ligand for the asialoglycoprotein receptor, to isolated parenchymal cells. The affinity for the asialoglycoprotein receptor significantly increased with increasing spacer length. *N*-[[[Tris-*O*-(β-D-galactopyranosyl)-3,6,9-trioxaundecanoxy]methoxy]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (**4e**), a cluster galactoside provided with a 20 Å spacer, possessed an at least 2000-fold higher affinity for the receptor than *N*-[[tris-*O*-(β-D-galactopyranosyl)methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (**4a**), a cluster galactoside lacking the spacer. It is concluded that vicinal galactosyl moieties within a cluster galactoside are more optimal recognized by the galactose binding sites of the asialoglycoprotein receptor upon proper spacing. The most potent galactoside, TG(20Å), may constitute an attractive targeting device for the specific delivery of drugs and/or genes to the parenchymal liver cell.

Introduction

Mammalian liver cells possess a specific membrane-bound receptor for glycoproteins containing terminal β-D-galactosyl or 2-acetamido-2-deoxy-β-D-galactopyranose (*N*-acetylgalactosamine) residues: the asialoglycoprotein receptor.[1,2] Uptake of glycoproteins by this receptor is both a high-affinity and high-capacity process. This feature, combined with its specific localization on the parenchymal liver cell, makes it an attractive target system for mediating the specific delivery of drugs and genes to this metabolically important cell. The availability of a potent synthetic ligand for the asialoglycoprotein receptor may facilitate the further development of a vector for the targeting of drugs and genes to the parenchymal liver cell. Structure–activity studies of synthetic galactosides and isolated oligoantennary glycopeptides with the asialoglycoprotein receptor have provided some insight into the basic structural requirements for ligand recognition. The nature of the branching pattern of the oligosaccharide component as well as the distance between the galactosyl residues were suggested to be major factors determining the affinity of a ligand.[3–7] A clearcut hierarchy of affinity has been observed with tetra- > tri- ≫ bi- ≫ monoantennary galactosides, the so-called "cluster effect".[5] Ligand recognition is, to a lesser extent, affected by changes in the hydrophobicity and structure of the aglycon part of the galactoside.[5,7] To date, however, synthetic galactosides have been optimized with respect to their branching pattern in order to accomplish high-affinity binding to the asialoglycoprotein receptor.[5–9] The effect of a variation in distance between the vicinal galactose residues within a clustered galactoside is rather unexplored. We therefore focused on a potential gain in affinity, arising from an optimization of the distance between the galactose residues.

The high-affinity galactosides described in this study might prove valuable in optimizing the strategy for hepatotrophic drug and gene targeting.

Results and Discussion

Synthesis. The synthesis of the cluster galactosides included three stages. The first step involved the preparation of building blocks **1d** and **3b**. Tris(hydroxymethyl)aminomethane was coupled to *N*-(benzyl-

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Abbreviations: TG(4Å), *N*-[Tris[*O*-(β-D-galactopyranosyl)methyl]-methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (**4a**); TG(9Å), *N*-[tris[[[*O*-(β-D-galactopyranosyl)ethoxy]methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (**4b**); TG(10Å), *N*-[tris[[[*O*-(β-D-galactopyranosyl)propoxy]methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (**4c**); TG(13Å), *N*-[tris[[[*O*-(β-D-galactopyranosyl)-3-oxapentoxy]-methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (**4d**); TG(20Å), *N*-[tris[[[*O*-(β-D-galactopyranosyl)3,6,9-trioxaundecanoxyl]methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (**4e**); TG(4Å)C, *N*-[tris[*O*-(β-D-galactopyranosyl)methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)]succinyl]glycinamide, (**5**); NIS, *N*-iodosuccinimide; TfOH, trifluoromethanesulfonic acid; KO*t*Bu, potassium *tert*-butylate; ASOR, asialoorosomucoid.

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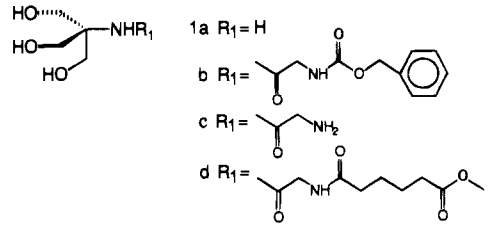
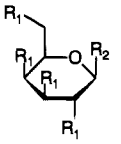
Scheme 1

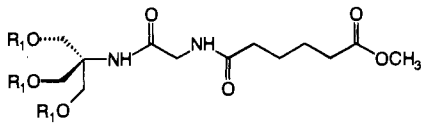


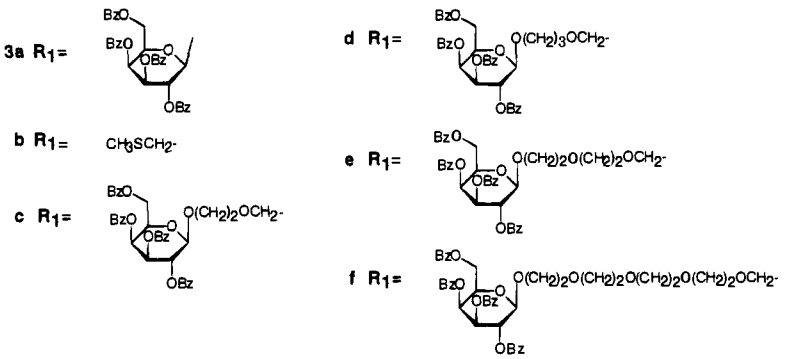
Scheme 2



2a R_1 = OAc R_2 = OAc
b R_1 = OBz R_2 = SC_2H_5
c R_1 = OBz R_2 = $O(CH_2)_2OH$
d R_1 = OBz R_2 = $O(CH_2)_3OH$
e R_1 = OBz R_2 = $O(CH_2)_2O(CH_2)_2OH$
f R_1 = OBz R_2 = $O(CH_2)_2O(CH_2)_2O(CH_2)_2O(CH_2)_2OH$

Scheme 3





Scheme 4

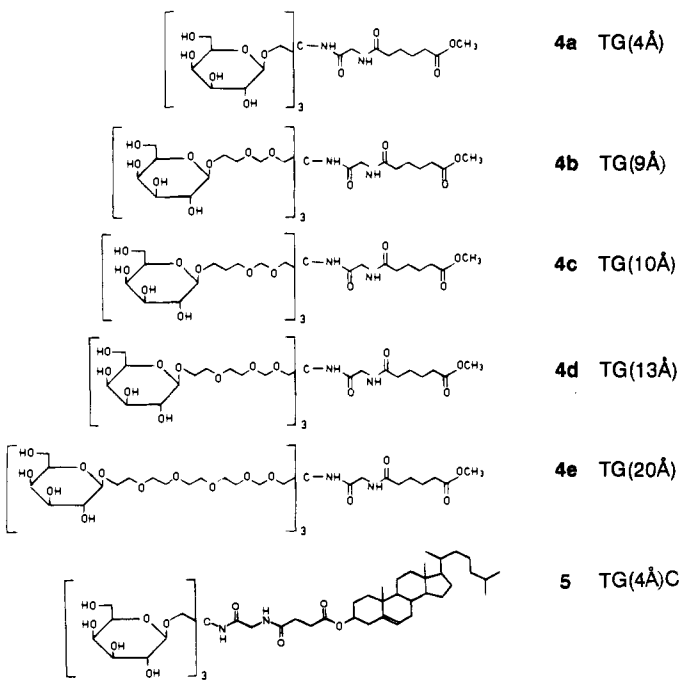


Figure 1. Chemical structures of the reaction intermediates (Schemes 1, 2, and 3) and the final products (Scheme 4) of the synthesis of cluster galactosides as described under Experimental Section.

oxycarbonyl)glycine by use of *N,N'*-dicyclohexylcarbodiimide to give the glycinamide derivative **1b** in 58% yield. Although this procedure was equally efficient as coupling using EEDQ as condensing agent described by Kempen et al.,[10] it was selected for its ease of workup.

After removal of the protective *N*-benzyloxycarbonyl group by catalytic hydrogenolysis using Pd/C, the resulting compound (**1c**) was directly elongated by reaction with succinimidyl-activated methyl adipate (**1b**) to yield **1d**. Efforts to thiomethylate **1d** according to the procedures of Kozikowski and Wu[11] or Pojer and Angyal[12] failed or gave only poor yields. Therefore, compound **1d** was thiomethylated according to the procedure of Medina et al.,[13] affording the trisubstituted thiomethyl derivative **3b** in a yield of 53% after purification by column chromatography.

Further, starting from a commercially available compound, **2a** was converted into the corresponding ethyl

2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-galactopyranoside, **2b**, in 81% yield. The fully protected thiogalactoside was derivatized at the 1-position with a 4-fold excess of some acceptor diols, i.e., ethylene glycol, 1,3-propanediol, di- and tetra(ethylene glycol), using *N*-iodosuccinimide (NIS) and catalytic amounts of trifluormethanesulfonic acid (TfOH).[14,15] Disubstituted diols, having higher R_f values than the monosubstituted diols, were formed as minor side products, but could be easily removed by column chromatography. Formation of the glycosidic linkage was in any case completely stereoselective (β-configuration) as ascertained by the characteristic large coupling constant (J > 7.7 Hz) between the H-1 and H-2 proton in the ^{1}H-NMR spectra as well as by the chemical shift of the anomeric C atom in the $^{13}C\{^1H\}$ NMR spectra (±100 ppm). Although the yield of NIS/TfOH-mediated coupling of acceptor diols (80–95%) was comparable to that reported for silver triflate-promoted derivatization of 2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl bromide with ethylene glycol,[16] this coupling procedure was selected for its ease and rapidity.

In the second stage the galactopyranosyl-derivatized compounds were introduced by glycosidation of the tris-(hydroxymethyl) dendrite. Thus, compound **2b** was coupled with the acceptor **1d** in the presence of NIS/TfOH. After workup, the isolated product **3a** was purified by a two-step procedure involving gel filtration over a LH20 column and Kieselgel column chromatography and was obtained in 36% yield. Both NMR analysis and mass-spectroscopical data of the tris-galactoside **3a** confirmed its chemical structure. This method was preferred to the conventional Koenigs–Knorr glycosylation under Helferich's conditions,[17] since it is far less laborious, faster, and gives comparable or even higher yields. In case of compound **3c–f** an alternative approach was used. Tris-galactosides provided with ethylene glycol, 1,3-propanediol, diethylene glycol, and tetra(ethylene glycol) spacers (**3c**, **3d**, **3e**, and **3f**, respectively) were synthesized as follows. Tris-(hydroxymethyl) dendrite was converted into a trismethyl(thiomethoxymethyl) dendrite, which operates as donor molecule for the NIS/TfOH-catalyzed glycosidation with hydroxyalkylated galactoside acceptors **2c–f**. This strategy was selected since it requires only one building block and thus leads to a more efficient synthetic route. Moreover, when tris(hydroxymethyl) was used as acceptor and the thiomethylated diol-derivatized 2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranose as donor for the NIS/TfOH-catalyzed glycosidation reaction, yields were considerably lower, which is probably due to intramolecular reaction of the activated thiomethylated compound. Therefore, TfOH/NIS-catalyzed glycosidation of **3b** with acceptor **2c–f** afforded a major component which was assigned to the trisgalactosylated derivative by NMR spectroscopy and mass spectrometry analysis together with a small amount of a minor component, probably the disubstituted galactoside derivative. Purification of the respective crude products in a two-step chromatographic procedure involving a LH20 and a Kieselgel column furnished **3c–f** in 24–53% yield. These moderate yields may arise from steric hindrance of the β-galactopyranosyl moieties at higher degrees of substitution. The present strategy represents a new and efficient synthetic pathway for preparation of more complex dendrites.

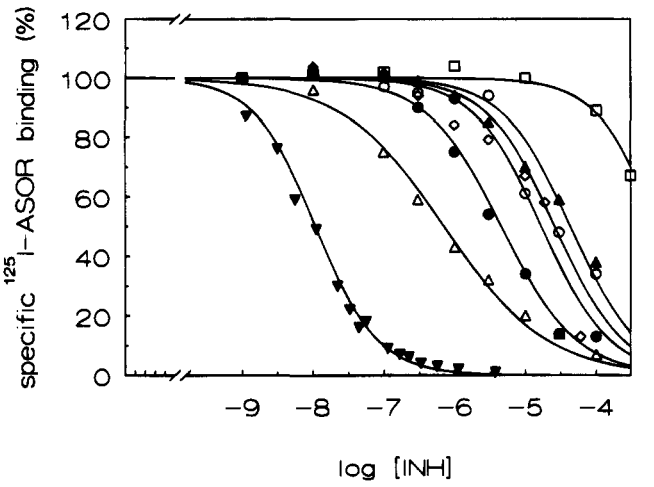


Figure 2. Competition curves of [^{125}I]ASOR binding to parenchymal cells for the synthesized cluster galactosides. Competition experiments were performed as follows. Freshly isolated rat parenchymal liver cells (10^6 cells/mL) were incubated for 2 h at 4 °C with a fixed concentration of [^{125}I]ASOR (5.5 or 11 nM) in the presence of the displacer, i.e. ASOR (▼), TG(4Å) (□), TG(4Å)C (◇), TG(9Å) (▲), TG(10Å) (●), TG(13Å) (○), and TG(20Å) (△) at concentrations, ranging from 10^{-9} to 10^{-3} M. 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). The inhibition curves were fitted according to a single site competition binding equation using a computerized nonlinear regression procedure (GraphPad).

The final stage involved the deprotection of the fully protected dendrites **3a** and **3c–f**. Complete debenzoylation was attained by treatment of **3a** and **3c–f** with KO–*t*–Bu in MeOH/1,4-dioxane (3/1, v/v) at a yield of approximately 80%. Following workup, each of the deprotected products, dissolved in water, was filtered and subsequently lyophilized. The residue was purified by chromatography over a high-resolution gel filtration column (S100, Pharmacia) with 0.1 M TEAB (pH = 7.0) as eluent. In this way, traces of partially debenzoylated and digalactosylated glycoside derivatives could be easily removed. On the basis of ^{13}C-NMR spectroscopy and mass spectrometry (PD), the isolated products were identified as tris-galactosylated glycosides **4a–e**. For compounds **4a–e** the elemental analysis matched theoretical within ±0.4%, which is indicative for high purity of the target compounds.

Biological Activity of the Synthesized Compounds 4a–e. In this study, we have synthesized a new series of oligoantennary galactosides with terminal galactose residues as potential high-affinity substrates for the hepatic asialoglycoprotein receptor. The various galactosides differ in the length of the intermediate spacer, which links the galactosyl moiety to the branching point of the cluster galactoside. The affinity of these compounds for the asialoglycoprotein receptor was monitored in an *in vitro* competition assay for [^{125}I]-ASOR binding to the parenchymal liver cell. All galactosides, except TG(4Å)C, displayed monophasic inhibition of [^{125}I]ASOR binding with a Hill coefficient close to unity, indicating fully competitive inhibition of [^{125}I]-ASOR binding (Figure 2). The affinity for the asialoglycoprotein receptor, expressed as the reciprocal of the K_i value, increased markedly with increasing spacer length

Table 1. Inhibition Constants for Competition of [^{125}I]ASOR Binding to Rat Parenchymal Liver Cells[a]

compound	K_i (μM)	pK_i ± SE
TG(4Å) (**4a**)	>390	3.41 ± 0.08
TG(9Å) (**4b**)	19	4.72 ± 0.12
TG(10Å) (**4c**)	1.2	5.91 ± 0.09
TG(13Å) (**4d**)	11	4.95 ± 0.26
TG(20Å) (**4e**)	0.2	6.68 ± 0.14
TG(4Å)C (**5**)	14	4.87 ± 0.16
ASOR	0.009	8.05 ± 0.12
orosomucoid	nd	
GalNAc	870	3.06 ± 0.14
GlcNAc	nd	

[a] Inhibition constants, K_i's, were calculated from inhibition curve data using a computerized non-linear regression procedure (Graph-Pad). K_i is denoted as mean ± standard error of three independent determinations; nd, not detectable.

(Table 1). TG(4Å) (**4a**), an analogue of previously synthesized TG(4Å) derivatives,[5,9,10,18] is only a poor displacer for [^{125}I]ASOR binding to both rat and rabbit parenchymal cells (K_i = 390 and 150 μM, respectively). The affinity of TG(4Å) is in the same order of magnitude as reported for the TG(4Å) analogue by Mary et al.[18] By contrast, an at least 2000-fold higher affinity was observed for the galactoside having the longest interchain, TG(20Å) (**4e**), which makes it one of the most potent triantennary β-D-galactopyranosyl-terminated dendrites that has been synthesized. Only 2-deoxy-*N*-acetyl-β-D-galactopyranose and β-D-lactose-terminated triantennary dendrites possess a higher affinity for this receptor.[7] TG(9Å) (**4b**), TG(10Å) (**4c**), and TG(13Å) (**4d**), respectively, displayed 20–300-fold higher, yet intermediate, affinities for the asialoglycoprotein receptor.

An intriguing feature involves the affinity of TG(10Å), which is 10-fold higher than both TG(9Å) and TG(13Å). This could arise from the enhanced hydrophobicity of the 1-propyl moiety of the terminal galactose groups of the cluster galactoside relative to the 1-ethyl group in TG(9Å) and TG(13Å). In fact, Connolly et al. have observed that a hydrophobic group directly attached to the β-D-galactoside may significantly increase the affinity of the galactoside for the asialoglycoprotein receptor.[5] *N*-[[Tris-*O*-(β-D-lactosyl)methyl]aminomethyl]-N^{α}-6-(*N*-benzyloxycarbonyl)hexanoylamine, the N-protected analogue of *N*-[[tris-*O*-(β-D-lactosyl)methyl]aminomethyl]-N^{α}-6-hexanoylamine displays a 10-fold increase in affinity relative to the mother compound. On the other hand, the propyl spacer may induce a more proper folding of the terminal galactosyl groups. Further study may clarify this apparent paradox.

To verify whether the cluster galactosides **4a**–**e** also inhibit asialoglycoprotein receptor-mediated uptake of [^{125}I]ASOR at 37 °C, the effect of the cluster galactosides on the rate of uptake of [^{125}I]ASOR by hepatocytes was determined. Lineweaver–Burk analysis of the substrate curves in the absence or presence of cluster galactoside revealed that all of the tested cluster galactosides displayed competitive inhibition of [^{125}I]ASOR uptake at 37 °C; the intercept on the 1/*v* axis of the Lineweaver–Burk plots was not affected by the presence of the cluster glycosides (Figure 3). From the plots, the apparent inhibition constant of the cluster galactosides could be calculated. The inhibition constants of the galactosides for [^{125}I]ASOR uptake at 37 °C and for [^{125}I]ASOR binding at 4 °C appeared to be essentially equal (Figure 4, correlation coefficient 0.973), suggesting that the enthalpy change of ligand binding to the

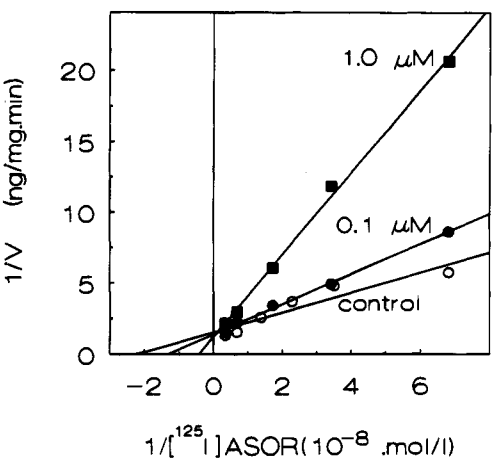


Figure 3. Typical double reciprocal plot (Lineweaver–Burk) of initial [^{125}I]ASOR uptake at 37 °C by hepatocytes in the absence or presence of TG(20Å) (**4e**). Parenchymal liver cells (10^6 cells/mL) were incubated for 10 min at 37 °C with 0 (○), 0.1 (●), and 1.0 μM TG(20Å) (■) and [^{125}I]ASOR (six concentrations ranging from 0 to 25 nM). After incubation, the cells were put on ice and the membrane-bound ASOR was removed by incubation for 10 min at 4 °C with 10 mM EDTA. Subsequently the cells were washed and cell-associated radioactivity was counted. Nonspecific uptake of [^{125}I]ASOR was determined in the presence of 100 mM GalNAc. Plotted is the reciprocal of specific [^{125}I]ASOR uptake versus the reciprocal of the rate of uptake.

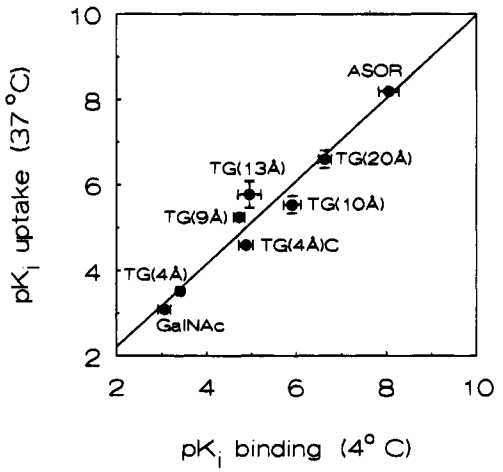


Figure 4. Correlation of the inhibition constants of cluster galactosides for [^{125}I]ASOR binding at 4 °C and the inhibition constants for [^{125}I]ASOR uptake at 37 °C. Inhibition constants (pK_i ± se) of ASOR, 2-acetamido-2-deoxy-β-D-galactopyranose (GalNAc), TG(4Å)C, TG(4Å), TG(9Å), TG(10Å), TG(13Å), and TG(20Å) were obtained from competition curves of [^{125}I]ASOR binding to hepatocytes at 4 ° C (see Table 1) and from Lineweaver-Burk plots of [^{125}I]ASOR uptake at 37 °C. The latter inhibition constants were calculated from the apparent Michaelis constant (K_m) in the absence and presence of TG(20Å) using the Cheng–Prussoff equation ($K_m^{app} = K_m(1 + [\text{inhibitor}]/K_i)$ as described in the Materials and Methods section.

asialoglycoprotein receptor is almost fully compensated by the change in binding entropy. More relevantly, however, it establishes that TG(20Å) (**4e**) also displays a high affinity for the asialoglycoprotein receptor under physiological conditions.

An attempt was made, using molecular modeling to correlate the observed increase in affinity, to change in the molecular characteristics of the synthesized cluster galactosides (Table 2). It appeared that an expansion of the interchain is accompanied both by an increase in

Table 2. Molecular Characteristics of the Various Cluster Galactosides as Determined Using Molecular Modeling

compound	spacer	length,[a] Å	distance,[b] Å
TG(4Å) (**4a**)	methyl	3.7	5.9
TG(9Å) (**4b**)	ethoxymethoxymethyl	9.2	14.2
TG(10Å) (**4c**)	propoxymethoxymethyl	10.4	16.5
TG(13Å) (**4d**)	3-oxapentoxymethoxymethyl	12.6	19.4
TG(20Å) (**4e**)	3,6,9-trioxaundecanoxymethoxymethyl	19.5	31.5

[a] Maximal spacer length, defined as being the distance between the anomeric center of the galactosyl and the branching point (in Å). [b] Distance between the anomeric centers of two vicinal galactosyls within a dendrite, configured in the maximal extended conformation (in Å).

the distance between two vicinal galactosyls and by an increase in flexibility. As a consequence, the β-galactosyls within the dendrite can scan ample conformational space without a significant change in intramolecular energy. It may be anticipated that, particularly in the case of TG(13Å) (**4d**) or TG(20Å) (**4e**), and under physiological hydrated conditions, the fully extended conformation will be a feasible one. Be that so, a tentative estimate can be given for the minimal distance, enabling appropriate binding of the separate vicinal β-galactopyranosyl moieties within the cluster galactoside. A distance of 14 (TG(10Å)) to 20Å (TG(13Å)) permits intermediate affinities, whereas a 32 Å spacing as in TG(20Å) will ensure high-affinity recognition by the asialoglycoprotein receptor. However, naturally occurring galactose-exposing glycopeptides and glycoproteins display affinities of up to 5 nM. This implies that even TG(20Å) may still be the subject of additional improvement arising from an optimalization of the aglycon part of the cluster galactoside. It still remains to be clarified whether the aforementioned additional increase in affinity can accomplished by further elongation of the tether connecting the terminal galactosides with the branching point. Since 2-deoxy-*N*-acetyl-β-D-galactosamine and β-D-lactose-terminated dendrites display affinities that are consistently higher than that of β-D-galactose-terminated dendrites, we anticipate that a potential gain in affinity can be attained by appropriate modification of other determinants like glucon structure and aglycon composition near the terminal glycoside.

In conclusion, a series of triantennary cluster galactosides has been prepared by glycosidation of thiomethylated alcohols. Competition with [^{125}I]ASOR binding studies demonstrated that the potency of a triantennary cluster galactoside for the asialoglycoprotein receptor strongly correlated with the length of the spacer between the separate galactosyl residues and the branching point of the galactoside. TG(20Å) (**4e**), provided with a 20 Å tetra(ethylene glycol) spacer, displayed an at least 2000-fold higher affinity for the asialoglycoprotein receptor as compared to TG(4Å) (**4a**), which lacks the spacer. Preliminary in vitro competition studies of lactosylated ^{125}I-labeled low-density lipoprotein binding to isolated Kupffer cells showed that the affinity toward the likewise galactose-recognizing galactose/fucose receptor on the hepatic Kupffer cell[19-21] was not altered upon elongation of the spacer of the galactoside from 4 to 20 Å.[22] This suggests that besides the affinity, the cellular specificity of the galactoside for the asialoglycoprotein receptor on parenchymal liver cells is also increased upon elongation of the spacer. Consequently, TG(20Å) will constitute an attractive carrier to specifically target drugs and genes to the parenchymal liver cells. Further studies on the potential use of TG(20Å) or TG(20Å) derivatives in the development of such a drug delivery system are currently underway.

Experimental Section

Chemicals and Solvents. 1,2,3,4,6-Penta-*O*-acetyl-β-D-galactopyranose (**2a**), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), *N*-hydroxysuccinimide, *N*-iodosuccinimide (NIS), trifluoromethanesulfonic acid (TfOH), 1-methyl adipate, ethanethiol, and dimethyl sulfide were purchased from Aldrich, potassium *tert*-butylate and Dowex 50-WX from Fluka. 1,3-Propanediol, ethylene glycol, di(ethylene glycol), and tetra(ethylene glycol), all obtained from Jansen Chimica, were dried over $MgSO_4$ and distilled under vacuum. Benzoyl chloride, and benzoyl peroxide from Baker were used without further purification. *N*,*N*-Dimethylformamide (DMF) was stirred for 16 h with CaH_2 (5 g/L) and then distilled under reduced pressure. Dichloroethane (DCE), dichloromethane (DCM), tetrahydrofurane (THF), 1,4-dioxane, acetone, and pyridine were refluxed for 16 h with CaH_2 (5 g/L), distilled, and stored over molecular sieves 4 Å, 4 Å, 5 Å, 4 Å, 4 Å, and 5 Å, respectively. Diethyl ether (Et_2O) was dried by heating under reflux with P_2O_5 (30 g/L) for 2 h, distilled, and stored over molecular sieves 4 Å. Methanol (MeOH) was dried over magnesium methoxide, prepared in situ by iodine (catalytic)-activated magnesium curls (5 g/L) in methanol (200 mL, p.a. Baker), refluxed for 1 h, and distilled. In an analogous way, ethanol (EtOH) was dried over ethoxide and distilled after refluxing for 1 h. Acetonitrile (extra dry; DNA synthesis grade) was obtained from Biosolve Ltd. (Barneveld, The Netherlands) and stored over molecular sieves (4 Å). Triethylammonium bicarbonate buffer (TEAB, 2 M) was prepared by passing a stream of carbon dioxide gas through a ice-cold mixture of triethylamine (825 mL) and deionized water (2175 mL) until pH 7.0–7.5. Cation-exchange resin (pyridinium form) was prepared by treatment of resin (Dowex 50 WX-4, 100–200 mesh, H^+ form) with pyridine/water (1/4, v/v; 250 mL), followed by washing the resin with water until pH 7.0. All other solvents and chemicals were reagent grade. Evaporations were carried out under reduced pressure (15 or 0.5 mmHg) at bath temperatures below 50 °C. *N*-[[Tris-*O*-(β-D-galactopyranosyl)methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)-succinyl]glycinamide (TG(4Å)C, **5**) was prepared according to the procedure described by Kempen et al.[10]

Chromatography. Thin-layer chromatography (TLC) was performed using silica F_{254} preformed layers (0.1 mm) on a plastic backing (Schleicher & Schüll DC Fertigfolien F1500) in the following mobile phases: A, DCM; B, DCM/MeOH (96/4, v/v); C, DCM/MeOH (95/5, v,v); D, DCM/MeOH (90/10, v/v); E, DCM/acetone (95/5, v/v); F, MeOH; G, DCM/MeOH (98/2, v/v); and H, DCM/MeOH (80/20, v/v). Carbohydrates were visualized after being sprayed with 20% H_2SO_4 in MeOH and subsequent heating at 140–160 °C. Compounds containing unsaturated bonds were visualized under UV, after exposition to I_2, or after spraying with molybdate reagent (H_2O/concentrated H_2SO_4/$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$/$(NH_4)_6Ce(SO_4)_4{\cdot}2H_2O$) (90/10/2.5/1, v/v/w/w)) followed by heating at 120 °C; sulfur or nitrogen-containing compounds were visualized by using a ninhydrin spray, or after exposition to Cl_2 vapor for 1 min, and subsequent soaking of the TLC sheet in solution of 4,4'-bis(dimethylaminodiphenylmethane, prepared according to the method of von Arx et al.[23] Preparative column chromatography was performed on silica (200–400 mesh ASTM, Merck). Sephadex LH20 (Pharmacia), suspended in DCM/MeOH (1/1, v/v), was used for gel-exclusion chromatography. Column

fractions of these runs were analyzed by TLC. FPLC high-resolution gel filtration was done on a sephacryl S100 column (S100 HR HiLoad XK26 from Pharmacia). The eluent was on line monitored on UV_{254} adsorption (Uvicord LKB 2158) or refractive index (Diff. Refractometer LKB 2142).

Instrumental Analysis. Melting points (uncorrected) were determined on a Büchi capillary melting point meter. NMR spectra were determined at 200 MHz (^1H) or 50.5 MHz (^{13}C) with a JEC-980B spectrometer operating in the Fourier Transform (FT) mode. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. For ^{13}C-NMR spectra, proton noise decoupling was used. Mass spectra were obtained using a plasma desorption mass spectrometer (PD) using chemical ionization (Cl) or particle beam spectrometry (PB) using a nitrocellulose matrix.

Syntheses. *N*-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]-*N*$^{\alpha}$-(benzyloxycarbonyl)glycinamide (1a, Scheme 1 of Figure 1). To a solution of 2-hydroxy-1,1-bis(hydroxymethyl)-aminoethane (1.8 g, 15 mmol) and *N*-(benzyloxycarbonyl)-glycine (3.2 g, 15 mmol) in DMF (50 mL) was added *N,N'*-dicyclohexylcarbodiimide (3.7 g, 17 mmol), and the solution was stirred for 2 days at ambient temperature. The suspension was filtered and the filtrate concentrated to dryness. Purification of the residue by column chromatography over a Kieselgel column (150 g) with DCM/MeOH (9/1, v/v) as eluent afforded **1a** as a white powder: yield 2.25 g (7.1 mmol, 47%); mp 131 °C; R_f 0.33 (D); the analytical data (TLC, ^1H-and ^{13}C-NMR analysis) were in every aspect identical to those described for **1a** by Kempen et al.[10]

1-Methyl 6-Succinimidyl Adipate (1b, Scheme 1 of Figure 1). To a solution of 1-methyl adipate (1.6 g, 100 mmol) and *N*-hydroxysuccinimide (11.6 g, 100 mmol) in acetone (200 mL) was added *N,N'*-dicyclohexylcarbodiimide (22.8 g, 110 mmol). After stirring for 2 h at 20 °C, the reaction mixture was filtered. The filtrate was allowed to stand for 2 h at -20 °C to precipitate the remaining traces of *N,N'*-dicyclohexylurea. The filtrate was evaporated to dryness, and the residue was purified by column chromatography over Kieselgel (150 g) using DCM/MeOH (95/5, v/v) as eluent. The appropriate fractions, containing the product, were pooled and concentrated in vacuo, yielding **1b** as a white powder: yield 24.3 g (94 mmol, 94%); R_f 0.73 (B), 0.59 (G); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 178.5 (C=O, OSu), 174.2 and 173.9 (2 × C=O, adipyl), 52.0 (N-C1, OSu), 42.5 (OCH_3), 34.3 (C5-adipyl), 30.4 (C2-adipyl), 29.9 (C3/C4-OSu), 26.1 (C3-adipyl), 25.4 (C4-adipyl).

***N*-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]-*N*$^{\alpha}$-[1-(6-methyladipyl)]glycinamide (1d, Scheme 1 of Figure 1).** Compound **1a** (15.6 g, 50 mmol) was dissolved in EtOH/MeOH (1/1, v/v; 200 mL), and the solution was dispersed with N_2. Pd/C (200 mg) was added, and the solution was shaken at 20 °C under a H_2 atmosphere (1 atm). After 2 h, the catalyst was removed by filtration, the filtrate concentrated in vacuo, and the oily residue (**1c**) used for the next step without further purification. Crude **1c** (8.1 g, 45 mmol) and compound **1b** (12.4 g, 50 mmol) in DMF (100 mL) were reacted for 3 h at 20 °C and, subsequently, for 18 h at 4 °C. The reaction mixture was filtered, and the filtrate was evaporated to dryness. The residue was purified by column chromatography over a Kieselgel column (200 g) eluted with DCM/MeOH (8/2, v/v). The collected fractions were concentrated to afford **1d** as a white amorphous solid: yield 12.8 g (40 mmol, 89%); R_f 0.40 (H); ^1H NMR ($CDCl_3$) δ 7.17 (t, J = 5.5 Hz, 1H, NH-Gly), 5.49 (s, 1H, NH-tris), 4.81 (t, J = 5.7 Hz, 3H, 3 × OH), 3.87 (d, J = 5.5 Hz, 2H, $CH_2\alpha$-Gly), 3.72 (d, J = 5.7 Hz, 6H, 3 × CH_2O), 3.65 (s, 3H, OCH_3), 2.32 (t, $J_{2,3} = J_{4,5}$ = 7.1 Hz, 4H, $CH_2$2- and $CH_2$5-adipyl), 1.64 (dd, $J_{3,4}$ = 3.5 Hz, $J_{2,3} = J_{4,5}$ = 6.9 Hz, 4H, $CH_2$3- and $CH_2$4-adipyl); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 176.3−172.2 (3 × C=O, 2 × amide and 1 × ester), 63.4 (C_{quat}-tris), 62.1 (CH_2-tris), 52.0 (Cα-Gly), 43.9 (OCH_3), 36.3 (C2-adipyl), 34.4 (C5-adipyl), 26.0 (C3-adipyl), 25.4 (C4-adipyl); mass (El) 320 (M^+).

Ethyl 2',3',4',6'-Tetra-*O*-benzoyl-1-thio-β-D-galactopyranoside (2b, Scheme 2 of Figure 1).[16] Compound **2a** (7.8 g, 20 mmol) was dried with and dissolved in DCE (75 mL). The mixture was cooled to 0 °C, and ethanethiol (1.63 mL, 22 mmol) was added. The reaction was initiated by dropwise addition of $SnCl_4$ (0.35 mL, 3.0 mmol), and the mixture was allowed to react for 1 h. The solution was successively washed with an aqueous KF (1M, 2 × 50 mL) and an aqueous $NaHCO_3$ solution (10% w/v, 2 × 50 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. The residue was dissolved in MeOH (100 mL), and KO-*t*-Bu (0.5g, 4 mmol) was added. After stirring for 1 h at 20 °C, the solution was neutralized by addition of Dowex pyridinium cation-exchange resin, filtered, and concentrated under reduced pressure. The crude β-D-galactopyranose derivative, dried by repeated evaporation with pyridine (2 × 20 mL, was dissolved in pyridine (50 mL), and benzoyl chloride (12.5 mL, 120 mmol) was added. After stirring for 1 h at 20 °C, the reaction was quenched with H_2O and the mixture concentrated. The residue was dissolved in DCM (50 mL) and washed with H_2O (50 mL) and an aqueous $NaHCO_3$ solution (10% w/v, 50 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated to afford **2b** as a white foam: yield 10.3 g (16 mmol; 81%); R_f 0.68 (E), 0.88 (C), and 0.72 (A); ^1H NMR ($CDCl_3$) δ 8.10−7.28 (m, 20H, H aromatic, benzoyl), 6.08 (d, $J_{4,3}$ = 3.1 Hz, 1H, H4-Gal), 5.91 (dd, $J_{2,1}$ = 8.8 Hz, $J_{2,3}$ = 9.5 Hz, 1H, H2-Gal), 5.78 (dd, $J_{3,4}$ = 3.2 Hz, $J_{2,3}$ = 9.9 Hz, 1H, H3-Gal), 4.91 (d, $J_{1,2}$ = 8.8 Hz, 1H, H1-Gal, β-configuration), 4.52−4.38 (m, 3H, H6, H6'-Gal and H5-Gal), 2.87 (q, J = 7.3 Hz, 2H, CH_2S), 1.33 (t, J = 7.5 Hz, 3H, CH_3C); $^{13}C\{^1H\}$ NMR (DMSO-d_6) δ 165.6−164.4 (C=O, benzoyl), 133.5−128.0 (C aromatic, benzoyl), 99.5 (C1-Gal, β-configuration), 71.5 (C5-Gal), 7.12 (C3-Gal), 68.8 (C4-Gal), 67.7 (C2-Gal), 62.2 (C6-Gal), 23.7 (CH_2S), 16.0 (CH_3C); mass (Cl) 658 $(M + NH_4)^+$.

General Procedure for the Synthesis of Hydroxy(oxa)-alkylated β-D-Galactopyranosyl Derivatives 2c−f (Scheme 2 of Figure 1). Typically, ethylene glycol (310 mg, 5 mmol) and **2b** (1.28 g, 2 mmol) was dried with DCE (25 mL) and dissolved in DCE/Et_2O (1/1 v/v; 10 mL). After addition of molecular sieves (300 mg; 3 Å), the mixture was stirred for 5 min at 20 °C and then cooled in an ice bath. To this mixture, a solution of NIS (560 mg, 2.5 mmol) and TfOH (22 μL, 0.25 mmol) in DCE/Et_2O (10 mL, 1/1, v/v) was added, and the reaction mixture was stirred for 5 min at 0 °C. The solution was filtered, successively washed with an aqueous $Na_2S_2O_3$ solution (1M, 25 mL) and an aqueous $NaHCO_3$ solution (10% w/v, 25 mL), dried over $MgSO_4$, filtered, and concentrated to dryness. The crude product was purified by Kieselgel column chromatography (50 g) with an elution gradient from 0 to 5% MeOH in DCM. The product, 2-hydroxyethyl 2',3',4',6'-tetra-*O*-benzoyl-β-D-galactopyranoside (**2c**), was isolated as a white foam: yield 1.03 g (1.6 mmol, 80%); R_f 0.59 (B); ^1H NMR ($CDCl_3$) δ 8.11−7.17 (m, 20H, H aromatic, benzoyl), 6.06 (d, $J_{3,4}$ = 3.3 Hz, 1H, H4-Gal), 5.86 (dd, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 10 Hz, 1H, H2-Gal), 5.70 (dd, $J_{2,3}$ = 10 Hz, $J_{3,4}$ = 3.3 Hz, 1H, H3-Gal), 4.94 (d, $J_{1,2}$ = 7.7 Hz, 1H, H1-Gal, β-configuration), 4.64 (dd, $J_{5,6}$ = 7.8 Hz, $J_{6,6'}$ = 11 Hz, 1H, H6-Gal), 4.47 (m, 2H, H6'-Gal and H5-Gal), 3.92 (m, 2H, CH_2OH), 3.63 (bs, 2H, CH_2O), 2.96 (bs, 1H, OH); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 165.2-165.7 (4 × C=O, benzoyl), 133.4−128.0 (C aromatic, benzoyl), 101.4 (C1-Gal, β-configuration), 72.4 (CO), 71.4 (C5-Gal), 71.1 (C3-Gal), 69.7 (C2-Gal), 68.0 (C4-Gal), 62.5 (C6-Gal), 61.9 (COH); mass (PB) 658 $(M + NH_4)^+$.

In a similar way, compounds **2d** to **2f** were synthesized using 1,3-propanediol (0.38 g), di(ethylene glycol) (0.53 g), and tetra(ethylene glycol) (0.97 g), respectively.

3-Hydroxypropyl 2',3',4',6'-tetra-*O*-benzoyl-β-D-galactopyranoside (2d, Scheme 2 of Figure 1): yield 1.09 g (1.67 mmol, 83%); R_f 0.31 (A), 0.63 (C); ^1H NMR ($CDCl_3$) δ 8.19−7.23 (m, 20H, H-aromatic, benzoyl), 6.04 (d, $J_{3,4}$ = 3.5 Hz, 1H, H4-Gal), 5.80 (dd, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 11 Hz, 1H, H2-Gal), 5.67 (dd, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.5 Hz, 1H, H3-Gal), 4.90 (d, $J_{1,2}$ = 7.7 Hz, 1H, H1-Gal, β-configuration), 4.56 (dd, $J_{5,6}$ = 6.8 Hz, $J_{6,6}$ = 10 Hz, 1H, H6-Gal), 4.49 (m, 2H, H6'- and H5-Gal), 3.70 (t, J = 3.0 Hz, 2H, $CH_2$3-propyl), 3.66 (t, J = 3.5 Hz, 2H, $CH_2$1-propyl), 3.01 (bs, 1H, OH), 1.81 (m, 2H, $CH_2$2-propyl); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 165.7, 165.4, 165.4 and 165.3 (4 × C=O, benzoyl), 133.4−128.0 (C2−C6, C-aromatic, benzoyl), 128.9 (C1, C-aromatic, benzoyl), 101.4 (C1-Gal, β-configuration), 71.4 (C5-Gal), 71.2 (C3-Gal), 69.7 (C2-Gal), 68.0 (C4-Gal), 62.5 (C6-Gal), 61.9 (C3-propyl), 59.3 (C1-propyl), 32.0 (C2-propyl); mass (PB) 672 $(M + NH_4)^+$.

5-Hydroxy-3-oxapentyl 2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-galactopyranoside (2e, Scheme 2 of Figure 1): yield 1.27 g (1.86 mmol, 93%); R_f 0.18 (A), 0.58 (C); ^{1}H NMR ($CDCl_3$) δ 8.01−7.17 (m, 20H, H-aromatic, benzoyl), 6.02 (d, $J_{3,4}$ = 3.3 Hz, 1H, H4-Gal), 5.72 (dd, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 11 Hz, 1H, H2-Gal), 5.64 (dd, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.3 Hz, 1H, H3-Gal), 4.79 (d, $J_{1,2}$ = 7.7 Hz, 1H, H1-Gal, β-configuration), 4.53 (dd, $J_{5,6}$ = 6.3 Hz, $J_{6,6'}$ = 10 Hz, 2H, H6-Gal), 4.43 (m, 2H, H6′- and H5-Gal), 3.65 (m, 2H, $CH_2$5-oxapentyl), 3.58 (s, 6H, $CH_2$1, $CH_2$2, and $CH_2$4 from oxapentyl), 2.94 (bs, 1H, OH); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 165.2−165.7 (4 × C=O benzoyl), 133.4−128.0 (C2−C6, C aromatic, benzoyl), 128.8 (C1, C aromatic, benzoyl), 101.4 (C1-Gal, β-configuration), 71.4 (C5-Gal), 71.0 (C3-Gal), 69.7 (C2-Gal), 68.0 (C4-Gal), 62.4 (C6-Gal), 61.9 (C5-oxapentyl), 61.1−61.3 (C from diethylene glycol); mass (PB) 702 (M + NH_4)$^+$.

11-Hydroxy-3,6,9-trioxaundecanyl 2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-galactopyranoside (2f, Scheme 2 of Figure 1): yield 1.47 g (1.9 mmol, 95%); R_f 0.09 (A), 0.43 (C); ^{1}H NMR ($CDCl_3$) δ 8.15−7.20 (m, 20H, H aromatic, benzoyl), 6.08 (d, $J_{3,4}$ = 3.5 Hz, 1H, H4-Gal), 5.78 (dd, $J_{1,2}$ = 7.5 Hz, $J_{2,3}$ = 10 Hz, 1H, H2-Gal), 5.67 (dd, $J_{2,3}$ = 10 Hz, $J_{3,4}$ = 3.5 Hz, 1H, H3-Gal), 4.89 (d, $J_{1,2}$ = 7.5 Hz, 1H, H1-Gal, β-configuration), 4.60 (dd, $J_{5,6}$ = 7 Hz, $J_{6,6}$ = 11 Hz, 1H, H6-Gal), 4.46 (m, 2H, H6′- and H5-Gal), 3.67 (t, 2H, $CH_2$11-trioxaundecanyl), 3.63 (s, 14H, remaining CH_2 from tetra(ethylene glycol)s), 3.10 (bs, 1H, OH); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 165.6−166.0 (4 × C=O benzoyl), 133.4−128.0 (C2−C6, C aromatic, benzoyl), 128.9 (C1, C aromatic, benzoyl), 101.4 (C1-Gal, β-configuration), 71.4 (C5-Gal), 71.1 (C3-Gal), 69.7 (C2-Gal), 68.0 (C4-Gal), 62.5 (C6-Gal), 61.9 (C11-trioxaundecanyl), 61.3−61.5 (C from tetra(ethylene glycol)); mass (PB) 790 (M + NH_4)$^+$.

***N*-[[Tris-*O*-(2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-galactopyranosyl)methyl]methyl]-N^α-[1-(6-methyladipyl)]glycinamide (3a, Scheme 3 of Figure 1).** Compound **2b** (512 mg, 0.8 mmol) and **1d** (64 mg, 0.2 mmol) were dissolved in DCE (25 mL). After removal of the solvent under reduced pressure, the residue was dissolved in DCE/Et_2O (1/1, v/v; 10 mL), molecular sieves (300 mg, 3 Å) were added, and the suspension was stirred for 15 min at 0 °C under N_2. To this mixture was added dropwise a solution of NIS (0.18 g, 0.8 mmol) and TfOH (12 mg, 0.08 mmol) in DCE/Et_2O (8 mL; 1/1, v/v). After 5 min, the reaction was quenched by addition of pyridine (0.2 mL). The suspension was filtered and washed with an aqueous $Na_2S_2O_3$ solution (1 M, 25 mL) and an aqueous $NaHCO_3$ solution (10%, w/v; 25 mL). The organic phase was dried over $MgSO_4$ and concentrated. The crude product was isolated by gel exclusion over a LH20 column (300 g, 75 × 2.5 cm) and subsequent chromatography of the front peak fractions over a Kieselgel column (50 g) eluted with MeOH/DCM (1/20, v/v). The fractions containing the main product were combined and concentrated to afford **3a** as a while foam: yield 148 mg (72 μmol, 36%); R_f 0.74 (E); ^{1}H NMR ($CDCl_3$) δ 8.07−7.20 (m, 60H, H aromatic, benzoyl), 5.84 (d, $J_{3,4}$ = 3.1 Hz, 3H, 3 × H4-Gal), 5.59 (d, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 10 Hz, 3H, 3 × H2-Gal), 5.41 (dd, $J_{2,3}$ = 10 Hz, $J_{4,3}$ = 3.3 Hz, 3H, 3 × H3-Gal), 4.54 (dd, $J_{5,6}$ = 6.3 Hz, $J_{6,6'}$ = 10 Hz, 3H, 3 × H6-Gal), 4.35 (dd, $J_{5,6}$ = 6.5 Hz, $J_{4,5}$ = 6.0 Hz, 6H, 3 × H5- and 3 × H6′-Gal), 4.11 (d, $J_{2,1}$ = 7.7 Hz, 3H, 3 × H1-Gal, β-configuration), 3.89 (d, J = 5.9 Hz, 2H, CH_2α-Gly), 3.75 (s, 6H, 3 × CH_2O-alkyl), 3.63 (s, 3H, OCH_3), 2.29 (t, $J_{2,3}$ = 7.0 Hz, 2H, CH_2, C2-adipyl), 2.17 (t, $J_{4,5}$ = 7.1 Hz, 2H, CH_2, C5-adipyl), 1.62 (dd, $J_{2,3}$ = $J_{4,5}$ = 7.1 Hz, 4H, CH_2, C3,4-adipyl); ^{13}C{^{1}H} ($CDCl_3$) δ 172.3 (C=O, amide), 168.6 (C=O, ester), 164.8−165.5 (C=O, benzoyl), 133.3−128.2 (C2−C6, C aromatic, benzoyl), 129.3 (C1, C aromatic, benzoyl), 101.7 (C1-Gal, β-configuration), 71.2 (C6-Gal), 71.1 (C3-Gal), 69.8 (C2-Gal), 67.8 (C4-Gal), 68.7 (C5-Gal), 61.5 (CH_2-tris), 59.7 (C_{quat}-tris), 51.9 (Cα-Gly), 41.4 (O-CH_3), 35.6 (C5-adipyl), 33.7 (C2-adipyl), 24.7 and 24.4 (C4,3-adipyl); mass (PD) 2060.9 (M + Li^+) and 2077.7 (M + Na^+).

***N*-[Tris[[(methylthio)methoxy]methyl]methyl]-N^α-[1-(6-methyladipyl)]glycinamide (3b, Scheme 3 of Figure 3).** Compound **1d** (0.96 g, 3.0 mmol) was dried with and dissolved in acetonitrile (30 mL). The mixture was cooled, and dimethyl sulfide (4.6 mL, 54 mmol) and benzoyl peroxide (6.5 g, 27 mmol) were successively added. After the mixture was stirred for 3 h at 0 °C, EtOAc (20 mL) was added and the reaction mixture washed twice with aqueous NaOH solution (0.1 N, 25 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated to dryness. The residue was purified by a two-step chromatographic procedure involving a LH20 column (70 × 2.5 cm) and a Kieselgel column (200 g) eluted with DCM. Crystallization from Et_2O/petroleum ether (40−60) yielded **3b** as white crystals: yield 1.0 g (2.0 mmol, 67%); R_f 0.67 (B); ^{1}H NMR ($CDCl_3$) δ 6.33 (bs, 1H, NH-Gly), 5.50 (s, 1H, NH-tris), 4.64 (s, 6H, 3 × SCH_2O), 3.89 (d, J = 6.1 Hz, 2H, CH_2α-Gly), 3.83 (s, 6H, 3 × OCH_2C), 3.67 (s, 3H, OCH_3), 2.34 (t, J = 6.9 Hz, 2H, $CH_2$5-adipyl), 2.25 (t, J = 3.5 Hz, 2H, $CH_2$2-adipyl), 2.14 (s, 9H, 3 × SCH_3), 1.67 (dd, J = 3.5 Hz, J = 6.9 Hz, 4H, $CH_2$3/$CH_2$4-adipyl); ^{13}C{^{1}H} ($CDCl_3$) δ 176.3 (C=O, amide), 175.4 (C=O, amide), 172.1 (C=O, ester), 75.4 (SCO), 66.5 (C_{quat}-tris), 63.5 (CH_2-tris), 52.0 (Cα-Gly), 44.0 (OCH_3), 36.3 (C5-adipyl), 34.3 (C2-adipyl), 26.1 (C4-adipyl), 25.4 (C3-adipyl), 13.6 (SCH_3); mass (PD) 523.5 (M + Na^+).

General Procedure for the Coupling of β-D-Galactopyranosyl Derivatives 2c to 2f to 3b (Scheme 3 of Figure 1). Typically, compound **3b** (100 mg, 0.2 mmol) and hydroxy-(oxa)alkyl-β-D-galactopyranol derivative (0.47 g of **2c**, 0.48 g of **2d**, 0.51 g of **2e** and 0.57 g of **2f**, respectively; 0.72 mmol) were dissolved in DCE (25 mL), and the solution was concentrated under reduced pressure. The residue was dissolved in DCE/THF (1/1 v/v, 4 mL), molecular sieves (300 mg, 4 Å) were added, and the suspension was stirred for 15 min under N_2. To this mixture, a solution of NIS (170 mg, 0.75 mmol) and TfOH (12 mg, 0.08 mmol) in DCE/THF (1/1 v/v, 8.5 mL) was added, and the mixture was stirred for 5 min at 0 °C. After addition of pyridine (1 mL), the suspension was filtered and the filtrate extracted with an aqueous $Na_2S_2O_3$ solution (1 M, 25 mL) and an aqueous $NaHCO_3$ solution (10% w/v; 25 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo. The respective crude products **3c**, **3d**, **3e**, or **3f** isolated after a two-step purification procedure involving an LH20 gel filtration column and a Kieselgel column chromatography (50 g) as described before for **3a**.

***N*-[Tris[[[*O*-(2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-galactopyranosyl)ethoxy]methoxy]methyl]methyl]-N^α-[1-(6-methyladipyl)]glycinamide (3c, Scheme 3 of Figure 1):** yield 109 mg (48 μmol, 24%); R_f 0.79 (D); ^{1}H-NMR ($CDCl_3$) δ 8.11−7.20 (m, 60H, H-aromatic, benzoyl), 6.01 (d, $J_{3,4}$ = 3.4 Hz, 3H, 3 × H4-Gal), 5.77 (dd, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 11 Hz, 3H, 3 × H2-Gal), 5.70 (m, 9H, OCH_2O and 3 × H3-Gal), 4.90 (d, $J_{1,2}$ = 7.7 Hz, 3H, 3 × H1-Gal, β-configuration), 4.68 (d, $J_{5,6}$ = 7 Hz, 3H, 3 × H6-Gal), 4.38 (m, 6H, 3 × H5- and 3 × H6′-Gal), 3.65 (s, 6H, OCH_2, ethylene glycol), 3.59 (s, 2H, CH_2α-Gly), 3.45 (s, 3H, OCH_3), 2.31 (t, $J_{2,3}$ = $J_{4,5}$ = 6.9 Hz, 4H, $CH_2$2/$CH_2$5-adipyl), 1.64 (dd, $J_{3,4}$ = 3.5 Hz, $J_{2,3}$ = $J_{4,5}$ = 6.9 Hz, 4H, $CH_2$3- and $CH_2$4-adipyl); ^{13}C{^{1}H} NMR ($CDCl_3$) δ 168.6−164.8 (C=O, 2 × amide, 1 × ester, 12 × benzoyl), 133.3−128.2 (C-aromatic, benzoyl), 101.6 (C1-Gal, β-configuration), 96.4 (OCO), 71.3 (C6-Gal), 71.1 (C3-Gal), 69.8 (C2-Gal), 67.8 (C4-Gal), 68.7 (C5-Gal), 61.9 (CH_2-tris), 61.5 (ethylene glycol), 59.7 (C_{quat}-tris), 51.4 Cα, Gly), 44.0 (OCH_3), 35.6 (C5-adipyl), 33.6 (C2-adipyl), 24.9 and 24.4 (C4/C3-adipyl); mass (PD) 2301.4 (M + Na^+).

***N*-[Tris[[[*O*-(2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-galactopyranosyl)propoxy]methoxy]methyl]methyl]-N^α-[1-(6-methyladipyl)]glycinamide (3d, Scheme 3 of Figure 1):** yield 131 mg (56 μmol, 28%); R_f 0.83 (B); ^{1}H-NMR ($CDCl_3$) δ 8.11−7.20 (m, 60H, H aromatic, benzoyl), 6.06 (d, $J_{3,4}$ = 2.9 Hz, 3H, 3 × H4-Gal), 5.90 (s, 6H, OCH_2O), 5.72 (dd, $J_{1,2}$ = 7 Hz, 3H, 3 × H2-Gal), 5.59 (m, 3H, 3 × H3-Gal), 4.91 (d, $J_{1,2}$ = 7.1 Hz, 3H, 3 × H1-Gal, β-configuration), 4.68 (m, 3H, 3 × H6-Gal), 4.41 (m, 6H, 3 × H5- and 3 × H6′-Gal), 3.64 (bs, 12H, $CH_2$1- and $CH_2$3-propyl), 3.59 (s, 2H, CH_2α-Gly), 3.45 (s, 3H, OCH_3), 2.40 (t, $J_{2,3}$ = $J_{4,5}$ = 7 Hz, 4H, $CH_2$2/$CH_2$5-adipyl), 1.80 (m, 6H, $CH_2$2-propyl), 1.68 (dd, $J_{3,4}$ = 3.5 Hz, $J_{2,3}$ = $J_{4,5}$ = 6.9 Hz, 4H, $CH_2$3/$CH_2$4-adipyl); mass (PD) 2341.1 (M + Na^+).

***N*-[Tris[[[*O*-(2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-galactopyranosyl)-3-oxapentoxy]methoxy]methyl]methyl]-N^α-[1-(6-methyladipyl)]glycinamide (3e, Scheme 3 of Figure 1):** yield 164 mg (68 μmol, 33%); R_f 0.44 (B); ^{1}H-NMR ($CDCl_3$) δ 8.10−7.09 (m, 60H, H-aromatic, benzoyl), 5.98 (d, $J_{3,4}$ = 3.5 Hz, 3H, 3 × H4-Gal), 5.85 (s, 6H, OCH_2O), 5.70 (dd, $J_{1,2}$ = 7.4

Hz, $J_{2,3}$ = 3.3 Hz, 3H, 3 × H2-Gal), 5.59 (m, 3H, 3 × H3-Gal), 4.86 (d, $J_{1,2}$ = 7.2 Hz, 3H, 3 × H1-Gal, β-configuration), 4.60 (m, 3H, 3 × H6-Gal), 4.45 (m, 6H, 3 × H5- and 3 × H6'-Gal), 3.65 (s, 18H, CH_2 from di(ethylene glycol)), 3.53 (s, 2H, CH_2α-Gly), 3.41 (s, 3H, OCH_3), 2.31 (t, $J_{2,3} = J_{4,5}$ = 6.9 Hz, 4H, $CH_2$2/$CH_2$5-adipyl), 1.64 (dd, $J_{3,4}$ = 3.5 Hz, $J_{2,3} = J_{4,5}$ = 6.9 Hz, 4H, $CH_2$3/$CH_2$4-adipyl); mass (PD) 2433.5 $(M + Na)^+$.

***N*-[Tris[[[*O*-(2',3',4',6'-tetra-*O*-benzoyl-β-D-galactopyranosyl)-3,6,9-trioxaundecoxy]methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (3f, Scheme 3 of Figure 1)**: yield 280 mg (105 μmol, 53%); R_f 0.47 (B); ^{1}H-NMR ($CDCl_3$) δ 8.14−7.06 (m, 60H, H-aromatic , benzoyl), 6.04 (d, $J_{3,4}$ = 3.1 Hz, 3H, 3 × H4-Gal), 5.96 (s, 6H, 3 × OCH_2O), 5.72 (dd, $J_{1,2}$ = 7.0 Hz, $J_{2,3}$ = 9.8 Hz, 3H, 3 × H2-Gal), 5.62 (dd, $J_{2,3}$ = 10 Hz, $J_{3,4}$ = 3.3 Hz, H3-Gal), 4.88 (d, $J_{1,2}$ = 6.7 Hz, 3H, 3 × H1-Gal, β-configuration), 4.69 (d, $J_{5,6}$ = 6.9 Hz, $J_{6,6'}$ = 10.5 Hz, 3H, 3 × H6-Gal), 4.38 (d, $J_{4,5}$ = 10 Hz, $J_{5,6}$ = 7.0 Hz, 6H, 3 × H5- and 3 × H6'-Gal), 3.70 (s, 42H, CH_2 from ethylene glycol), 3.61 (s, 2H, CH_2α-Gly), 3.47 (s, 3H, OCH_3), 2.27 (t, $J_{2,3} = J_{4,5}$ = 6.9 Hz, 4H, $CH_2$2/$CH_2$5-adipyl), 1.70 (dd, $J_{3,4}$ = 3.5 Hz, $J_{2,3} = J_{4,5}$ = 6.9 Hz, 4H, $CH_2$3/$CH_2$4-adipyl); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 165.9−158.6 (C=O, 2 × amide, 1 × ester, and 12 × benzoyl), 133.5−128.2 (C-aromatic, benzoyl), 101.7 (C1-Gal, β-configuration), 95.7 (OCO), 71.6 (C6-Gal), 71.2 (C3-Gal), 69.7 (C2-Gal), 69.1−69.3 (C from tetra(ethylene glycol)), 68.6 (C4-Gal), 68.7 (C5-Gal), 61.9 (CH_2OH), 61.5 (CH_2-tris), 58.9 (C_{quat}-tris), 51.4 (Cα, Gly), 42.9 (OCH_3), 35.6 (C5-adipyl), 33.7 (C2-adipyl), 24.8 and 24.6 (C4,3-adipyl); mass (PD) 2694 $(M + Na)^+$.

Deprotection of Benzoylated Compounds 3a and 3c−f. To compound **3a**, **3c**, **3d**, **3e**, or **3f** (50 μmol) in MeOH/1,4-dioxane (3/1, v/v; 10 mL) was added KO-*t*-Bu (60 mg), and the solution was stirred for 4 h at room temperature. The solution was neutralized by addition of Dowex 50 WX-4 pyridinium cation-exchange, filtered, and concentrated in vacuo. The residue was dissolved in H_2O and the solution filtered and lyophilized. The crude produce was purified over a sephacryl S100-column, using TEAB (0.1 M, pH = 7.0) as eluent. The peak fractions containing pure product were evaporated with MeOH/H_2O (1/1, v/v) and lyophilized.

***N*-[Tris[*O*-(β-D-galactopyranosyl)methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG(4Å); 4a, Scheme 4 of Figure 1)**: yield 30 mg (75%); R_f 0.42 (F); $^{13}C\{^1H\}$ NMR (D_2O) δ 176.1−171.6 (3 × C=O, amides and ester), 104.1 (C1-Gal, β-configuration), 75.7 (C5-Gal), 73.1 (C3-Gal), 71.3 (C2-Gal), 69.2 (C4-Gal), 68.2 (C6-Gal), 61.6 (CH_2-tris), 60.5 (C_{quat}-tris), 52.7 (OCH_3), 43.4 (Cα-Gly), 35.6 (C2-adipyl), 33.9 (C5-adipyl), 25.1 (C3-adipyl), 24.3 (C4-adipyl); mass (PD) 828.9 $(M + Na)^+$ ($M_{calculated}$ 803.8); V_{el} sephacryl S100 column, 259.2 mL (K_{av} = 0.82). Anal. ($C_{31}H_{54}N_2O_{22}$) C, H, N.

***N*-[Tris[[[*O*-(β-D-galactopyranosyl)ethoxy]methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG-(9Å)C; 4b, Scheme 4 of Figure 1)**: yield 42 mg (81%); R_f 0.29 (F); $^{13}C\{^1H\}$ NMR (D_2O) δ 177.6−171.4 (3 × C=O amides, ester), 103.5 (C-1, β-Gal), 96.3 (OCO), 75.7 (C5-Gal), 73.3 (C3-Gal), 71.4 (C2-Gal), 70.3 (C1-ethyl), 70.1 (C2-ethyl), 69.3 (C4-Gal), 69.1 (C6-Gal), 67.3 (CH_2-tris), 61.5 (C_{quat}-tris), 52.1 (OCH_3), 44.1 (Cα-Gly), 35.9 (C2-adipyl), 34.1 (C5-adipyl), 25.7 (C3-adipyl), 24.0 (C4-adipyl); mass (PD) 1037.3 (+LiCl), 1056.2 $(M + Na)^+$ ($M_{calculated}$ 1028.6); V_{el} sephacryl S100 column, 245.6 mL (K_{av} = 0.75). Anal. ($C_{40}H_{72}N_2O_{28}$) C, H, N.

***N*-[Tris[[[*O*-(β-D-galactopyranosyl)propoxy]methoxy]methyl]methyl]-N^{α}-1(6-methyladipyl)]glycinamide (TG-(10Å); 4c, Scheme 4 of Figure 1)**: yield 43 mg (81%); R_f 0.38 (F); $^{13}C\{^1H\}$ NMR (D_2O) δ 177.6−171.4 (3 × C=O amides, ester), 103.5 (C1-Gal, β-configuration), 96.1 (OCO), 75.7 (C5-Gal), 73.4 (C3-Gal), 71.3 (C2-Gal), 68.6 (C1), 67.7 (C3-propyl), 69.2 (C4-Gal), 67.7 (C6-Gal), 66.8 (CH_2-tris), 61.3 (C_{quat}-tris), 51.8 (OCH_3), 46.9 (Cα-Gly), 35.8 (C2-adipyl), 34.1 (C5-adipyl), 25.9 (C3-adipyl), 29.5 (C2-propyl), 24.2 (C4-adipyl); mass 1098.1 $(M + Na)^+$ ($M_{calculated}$ 1070.6); V_{el} sephacryl S100 column, 243.2 mL (K_{av} = 0.73). Anal. ($C_{43}H_{78}N_2O_{28}$) C, H, N.

***N*-[Tris[[[*O*-(β-D-galactopyranosyl)-3-oxapentoxy]methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG(13Å); 4d, Scheme 4 of Figure 1)**: yield 45 mg (78%); R_f 0.21 (F); $^{13}C\{^1H\}$ NMR (D_2O) δ 171.0 (3 × C=O amides, ester), 103.4 (C1-Gal, β-configuration), 95.9 (OCO), 75.7 (C5-Gal), 73.4 (C3-Gal), 71.4 (C2-Gal), 70.3−2 (C from 3-oxapentanyl), 69.3 (C4-Gal), 69.1 (C6-Gal), 67.3 (CH_2-tris), 61.6 (C_{quat}-tris), 52.1 (OCH_3), 44.9 (Cα-Gly), 35.9 (C2-adipyl), 32.2 (C5-adipyl), 25.3 (C3-adipyl), 24.1 (C4-adipyl); mass 1186.3 $(M + Na)^+$ ($M_{calculated}$ 1160.7); V_{el} sephacryl S100 column, 238.5 mL (K_{av} = 0.71). Anal. ($C_{46}H_{84}N_2O_{31}$) C, H, N.

***N*-[Tris[[[*O*-(β-D-galactopyranosyl)-3,6,9-trioxaundecoxy]methoxy]methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG(20Å); 4e, Scheme 4 of Figure 1)**: yield 55 mg (77%); R_f 0.14 (F); $^{13}C\{^1H\}$ NMR (D_2O) δ 177.6−171.4 (3 × C=O amides, ester), 103.5 (C1-Gal, β-configuration), 96.0 (OCO), 75.7 (C5-Gal), 73.3 (C3-Gal), 71.5 (C2-Gal), 70.3−70.1 (C from 3,6,9-triaxoundecanyl), 69.8 (C4-Gal), 69.2 (C6-Gal), 67.3 (CH_2-tris), 61.8 (C_{quat}-tris), 52.0 (OCH_3), 43.4 (Cα-Gly), 37.7 (C2-adipyl), 35.9 (C5-adipyl), 26.0 (C3-adipyl), 25.7 (C4-adipyl); mass 1435.4 $(M + Li)^+$, 1448.1 $(M + Na)^+$ ($M_{calculated}$ 1424.9); V_{el} sephacryl S100 column, 229.9 mL (K_{av} = 0.66). Anal. ($C_{58}H_{108}N_2O_{37}$) C, H, N.

Isolation of Parenchymal Liver Cells. Male Wistar rats of approximately 250 g were anaesthetized by intraperitoneal injection of 20 mg of sodium pentobarbital. Parenchymal liver cells were isolated after a 20 min perfusion of the liver with collagenase (type IV, 0.05% w/v) at 37 °C by the method of Seglen,[24] modified as previously described.[25] Following perfusion, parenchymal cells were purified by differential centrifugation as described in detail elsewhere.[26] The obtained liver cells were ≥95% viable as judged by 0.2% trypan blue exclusion and ≥99% pure as judged by light microscopy.

Iodination of Asialoorosomucoid. Human orosomucoid was isolated and subsequently desialylated enzymatically as described.[27] The protein was radiolabeled with carrier free $Na^{125}I$ by the ICl method of McFarlane[28] as modified by Bilheimer et al.[29]

In Vitro Binding and Uptake Studies. Displacement of [^{125}I]ASOR binding to hepatocytes was determined as previously described.[19] In short, 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) in the presence or absence of unlabelled displacer at eight concentrations, ranging from 1 nM to 1 mM. After 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. Nonspecific binding was measured in the presence of 100 mM GalNAc.

The potency of cluster galactosides to inhibition [^{125}I]ASOR uptake by hepatocytes at 37 °C was studied as follows. Hepatocytes (1−1.5 × 10^6 cells) were incubated in 1 mL of the above medium for 10 min at 37 °C with [^{125}I]ASOR (six concentrations ranging from 0.5 to 20 nM) in the presence of a fixed concentration of unlabeled displacer. After incubation, the cells were put on ice and EDTA was added to a final concentration of 10 mM in order to remove membrane-bound [^{125}I]ASOR. The cells were incubated for 10 min and subsequently washed as described above. Nonspecific uptake was determined in the presence of 100 mM GalNAc. From nonlinear regression analysis of the [^{125}I]ASOR substrate curves in the absence or in the presence of displacer (two concentrations) the Michaelis−Menten constants could be calculated. The inhibition constant of the cluster glycosides could be quantified from the apparent Michaelis constant K_m^{app} and using the Cheng−Prussoff equation $K_m^{app} = K_m(1 + [\text{inhibitor}]/K_i)$.[30] Protein contents were determined according to the method of Lowry, using BSA as the standard.[31]

Computational Methods. Displacement binding data were analyzed according to a single site model using a computerized nonlinear fitting program (Graph-Pad)[32] to calculate the K_i. Model building and energy minimalization studies were carried out using the software package BIOGRAF version 2.2 (Molecular Simulations, Sunnyvale, CA). All manipulations were performed on a Silicon Graphics 4D/25GT workstation. Throughout all the calculations default values for the various parameters in the BIOGRAF molecular mechanics (Dreiding[33]) force field were used. Conjugate gradient

energy minimizations were continued until the rms energy gradient was less than 0.1 kcal/mol.[34]

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