The Cholesterol Derivative of a Triantennary Galactoside with High Affinity for the Hepatic Asialoglycoprotein Receptor: a Potent Cholesterol Lowering Agent†

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Cholesterol-derivatized galactosides have been devised in order to induce liver uptake of lipoproteins via the galactose-recognizing asialoglycoprotein receptor in the liver. In this study we describe the derivatization of a newly developed triantennary cluster galactoside having high affinity for the asialoglycoprotein receptor, *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG(20Å)) with cholesterol. Hereto, TG(20Å) was coupled to glycine-(5-cholesten-3β-yl ester) in the presence of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate, affording *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-(5-cholesten-3β-yloxy)glycyl)adipyl]glycinamide (TG(20Å)C) in 46% yield. This compound is an amphiphilic, water-soluble compound. In aqueous solution it readily formed small micelles (4.9 ± 1.2 nm) consisting of approximately 20 molecules. Upon incubation with human serum, TG(20Å)C spontaneously incorporated into the most prominent serum lipoproteins, i.e., low-density lipoprotein (LDL) and high-density lipoprotein (HDL), thereby inducing an increase in buoyant density of these lipoproteins. The integrity of HDL and LDL, as judged from particle size analysis of both lipoproteins, was not altered by incubation with up to 0.33% of TG(20Å)C (w/v). Following intravenous bolus injection into rats, TG(20Å)C induced a dose-dependent decrease in the serum cholesterol content of maximally 44%, at a dose of 1.9 mg kg^{-1}. This makes TG(20Å)C at least 30-fold more effective than the previously developed *N*-[[tris-*O*-(β-D-galactopyranosyl)methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)succinyl]glycinamide (TG(4Å)C), provided with a cluster galactoside that displayed a 2000-fold lower affinity for the asialoglycoprotein receptor than TG(20Å). In conclusion, the hypocholesterolemic activity of a cholesterylated galactoside can be strongly enhanced by using a cluster galactoside with higher affinity for the asialoglycoprotein receptor.

Introduction

High plasma levels of the major vehicle for cholesterol transport in humans, the low-density lipoprotein (LDL), are correlated with an increased occurrence of atherosclerosis.[1,2] Therapy for hypercholesterolemia generally aims to block cholesterol synthesis or prevent readsorption of bile acids. This leads to an up-regulation of hepatic LDL receptors and thus an enhanced catabolism of LDL-derived cholesterol.[3,4] In general, FH patients[5] and patients with mutations in apolipoprotein B_{100}[6] do not sufficiently respond to this therapy, so expensive and invasive plasmapheresis is needed in order to lower the blood LDL levels.[7]

Within our laboratory, we have devised cholesterol-lowering drugs that induce hepatic uptake of lipoproteins via non-lipoprotein and, in particular, galactose-recognizing receptors (the so-called asialoglycoprotein receptor[8,9]) on the parenchymal liver cell. Bifunctional drugs, consisting of a moiety that associates with atherogenic lipoproteins and a second moiety that is recognized by the asialoglycoprotein receptor, have been developed, and the potentials of this approach for lowering serum LDL levels have been demonstrated.[10–15] A cholesterylated cluster galactoside, *N*-[[tris-*O*-(β-D-galactopyranosyl)methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)succinyl]glycinamide (TG(4Å)C), significantly reduced the serum cholesterol level in the rat.[11, 14, 15] However, a continuous intravenous infusion with relatively high doses of TG(4Å)C was required in order to accomplish a detectable therapeutic effect.[12] TG(4Å)C directed both LDL and HDL to the liver,[12,15] at which HDL was mainly taken up by parenchymal liver cells and LDL by Kupffer cells.[15] A monogalactosylated cholesterol derivative also directed the atherogenic LDL to hepatic Kupffer cells.[13,16] As only the parenchymal liver cell is anatomically coupled to bile secretion, this cell is the preferred site for direct and irreversible removal of cholesterol from the bloodstream.[12] Both the low efficacy and the stimulation of Kupffer cell-mediated uptake may be based on the rather low affinity of both (cluster) galactosides for the hepatic asialoglycoprotein receptor. Recently, we have synthesized a new series of triantennary cluster galactosides.[17] It was demonstrated that elongation of the spacer, connecting the separate β-galactose residues with the branching point of the cluster galactoside, leads to an increased affinity of the galactoside for the asialoglycoprotein receptor. Illustratively, TG(20Å), which has a 20 Å spacer, displayed an affinity of 200 ± 76 nM while TG(4Å), the

† Abbreviations: TG(20Å) (**2**), *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-methyladipyl)]-glycinamide; TG(20Å)C (**4**), *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-(5-cholesten-3β-yloxy)-glycyl)adipyl]glycinamide; TG(4Å)C (**5**), *N*-[[tris-*O*-(β-D-galactopyranosyl)-methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)succinyl]glycinamide; LDL, low-density lipoproteins; HDL, high-density lipoproteins.

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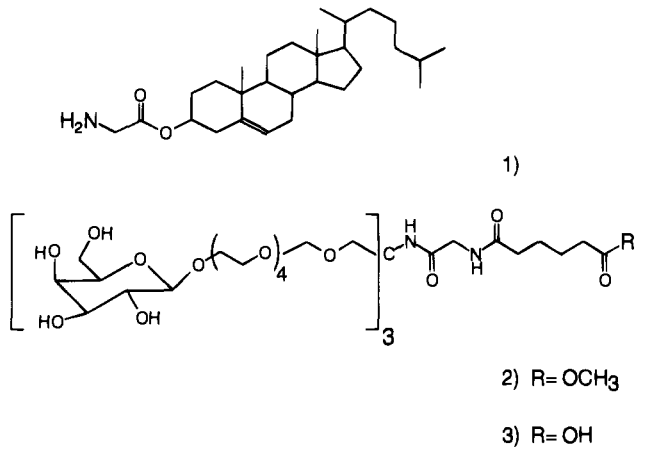

Figure 1. Chemical structures of the reaction intermediates of the synthesis of TG(20Å)C (**4**) as described in the Experimental Section.

triantennary galactoside component of TG(4Å)C provided with a 4 Å spacer, possessed a 2000-fold lower affinity for this receptor (K_i = 390 ± 77 μM).[17]

In the current study, the synthesis and physicochemical characterization of the cholesterol derivative of TG(20Å), *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-(5-cholesten-3β-yloxy)glycyl)adipyl]glycinamide (TG(20Å)C) is described. It is demonstrated that TG(20Å)C is at least 30-fold more effective in lowering serum cholesterol levels in the rat than the previously developed TG(4Å)C compound.

Results and Discussion

Syntheses. Recently, we have synthesized a series of triantenarry cluster galactosides with terminal β-D-galactopyranosyl moieties and we have determined the affinity of these galactosides for the hepatic asialoglycoprotein receptor using in vitro studies of [^{125}I]asialoorosomucoid binding (4 °C) to and uptake (37 °C) by isolated hepatocytes.[17] The inhibition constant of *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG(20Å); see Figure 1, **2**), a cluster galactoside provided with a 20 Å spacer between the galactosyl residues and the branching point, was 200 ± 76 nM at 4 °C and 250 ± 82 nM at 37 °C. This is 2000-fold higher than that of a previously synthesized triantennary cluster galactoside that lacks the spacer, *N*-[[tris-*O*-(β-D-galactopyranosyl)methyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG(4Å); K_i = 390 ± 77 and K_i = 300 ± 45 μm, respectively.[17] It was concluded that elongation of the spacer connecting the separate galactosyl residues with the branching point of the cluster galactoside is accompanied by a strong increase in the affinity of the cluster galactosides for the asialoglycoprotein receptor. The current study communicates on the synthesis of the cholesterol derivative of TG(20Å). TG(20Å) (see Figure 1, **2**) was first functionalized by demethylation of the terminal methyl adipate using Tesser's base (NaOH in H_2O/1,4-dioxane (3/1, v/v).[18] The resulting free acid was condensed with glycine-(5-cholesten-3β-yl ester) hydrotrifluoroacetate (see Figure 1, **1**) in the presence of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate and a catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine. In view of the large number of free hydroxyl groups within the compound, this method was preferred to conventional coupling methods using carbodiimides[19] or *N*-(ethoxycarbonyl)-3-ethoxy-1,2-dihydroquinoline.[20] The crude reaction mixture was chromatographed over a sephadex LH20 column and a Kieselgel 60 column, and the putative product, *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-(5-cholesten-3β-yloxy)glycyl)adipyl]glycinamide (TG(20Å)C; **4**; for the chemical structure, see Figure 2), was isolated in 46% yield. The mass spectrum (Figure 3), elemental analysis and TLC staining pattern, and $^{13}C\{^1H\}$ and ^1H NMR spectra were in agreement with the proposed structure of TG(20Å)C. The purity was estimated, on the basis of TLC analysis, elemental analysis, and mass spectroscopy, to be at least 95%.

Physicochemical Properties. TG(20Å)C is a highly amphiphilic compound. Size analysis of a solution of TG(20Å)C in PBS by photon correlation spectroscopy revealed that, in aqueous solution, TG(20Å)C readily forms micelles. The TG(20Å)C−micelle has an apparent radius of 4.9 ± 1.2 nm and consists of approximately 20 molecules of TG(20Å)C. This size compares well with that of TG(4Å)C micelles (5.0 ± 1.2 nm) and digitonin micelles (a structurally analogous spirostane-derivatized

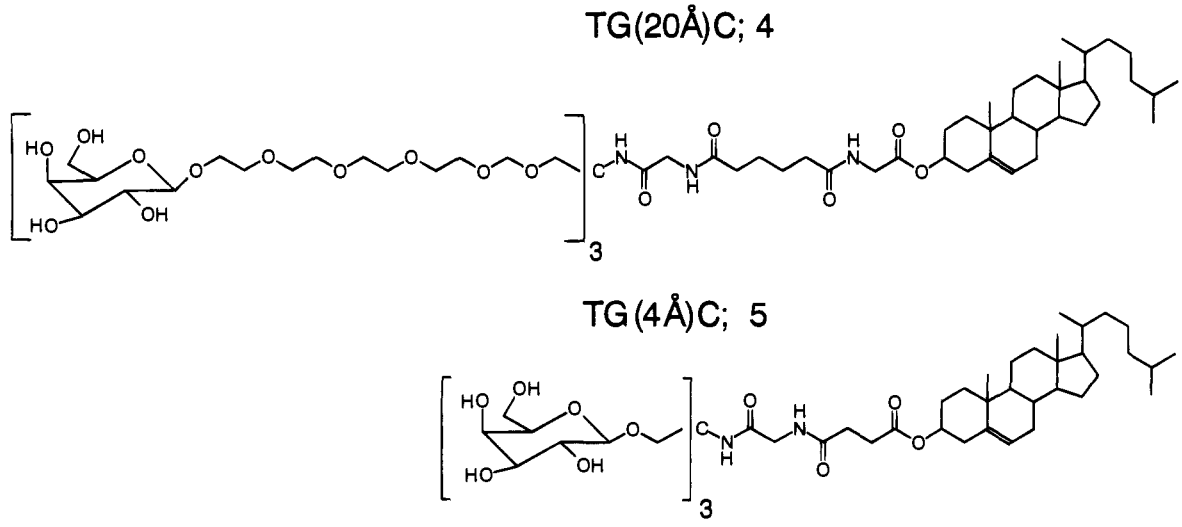

Figure 2. Chemical structures of TG(20Å)C (**4**) and TG(4Å)C (**5**).

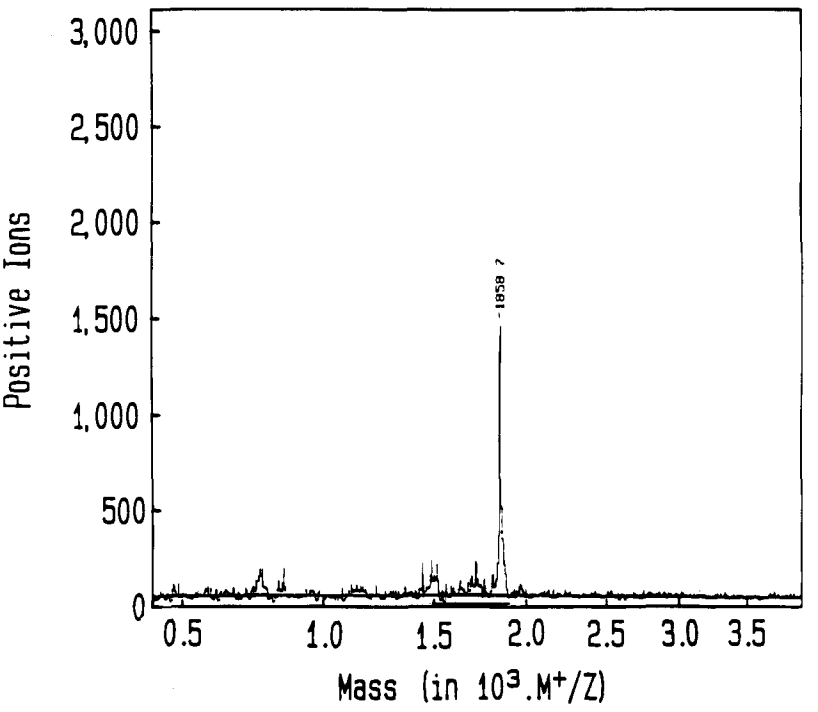

Figure 3. Mass spectrum of *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-(5-cholesten-3β-yloxy)glycyl)adipyl]glycinamide (TG(20Å)C, **4**).

oligosaccharide) (4.3 ± 0.7 nm). Its tendency to form micelles in aqueous solution renders TG(20Å)C water-soluble, despite of its bulky hydrophobic cholesterol moiety.

Interaction of TG(20Å)C with Lipoproteins from Human Serum. The interaction of the cholesterol-derivatized triantennary cluster galactosides with lipoproteins was studied by incubating human serum with 0.8 mM of TG(20Å)C or TG(4Å)C at room temperature. This enables a direct comparison with data obtained from previous studies using TG(4Å)C[11] or mono-Galchol.[13] The mixture was subjected to density-gradient ultracentrifugation in KBr/NaCl solutions, and the gradient was scanned for cholesterol and carbohydrate content. In the presence of TG(20Å)C or TG(4Å)C, the most prominent lipoproteins in human serum, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) have a significantly higher buoyant density (Figure 4). The observed shift in LDL density can be fully attributed to incorporation of TG(20Å)C and TG(4Å)C, having specific densities of 1280 and 1220 kg/m^3, respectively. Assuming 250 molecules of TG(4Å)C and 210 molecules of TG(20Å)C to incorporate into LDL (see below), this corresponds to a density shift from 1050 to approximately 1080 and 1090 kg/m^3, respectively. The effect of incorporation of both glycolipids on HDL density was significantly smaller owing to the small difference in densities between HDL and the glycolipids. The effect of TG(4Å)C was slightly less pronounced than that of TG(20Å)C. The total cholesterol content of these lipoproteins remained constant upon incubation with TG(20Å)C and TG(4Å)C (6.47 ± 0.20 μmol). Besides a peak at high buoyant density, arising from carbohydrate containing serum glycoproteins, two additional sugar peaks are observed in the sugar profile of the TG(4Å)C- and TG(20Å)C-treated sera. These peaks essentially coincided with the HDL and LDL peaks from the cholesterol profile, suggesting that both galactosides associate with these lipoproteins. From the carbohydrate content in the HDL and LDL fractions, the extent of incorporation of the cluster galactosides into these lipoproteins can be estimated. Approximately 57% and 43%, respectively of TG(4Å)C and 64% and 36%, respectively, of TG(20Å)C appeared to be associated with HDL and LDL (after correction for the glycoside background in both of the lipoprotein fractions). Assuming a LDL particle to contain 1800 cholesterol(ester) molecules and a HDL particle to contain 110 molecules of cholesterol(esters),[21] it can be calculated that about 246 molecules of TG(4Å)C and 213 molecules of TG(20Å)C are incorporated into a single molecule of LDL, whereas 43 molecules of TG(4Å)C and 49 molecules of TG(20Å)C are incorporated into a single molecule of HDL. TG(4Å)C, however, seems to display a slight preference for accumulation in LDL as compared to TG(20Å)C. Although care should be taken before extrapolating the results of the incorporation and integrity studies obtained at 20 °C to physiological conditions (37 °C); they probably are a good reflection of the physicochemical behavior of TG(20Å)C at 37 °C as the phase-transition temperature of lipoproteins is much higher (50–55 °C).

Integrity of HDL and LDL in the Presence of TG(20Å)C. From the above data it can be concluded that TG(20Å)C associates spontaneously into lipoproteins upon incubation with serum. The question rises whether the integrity of these lipoproteins is affected by incorporation of TG(20Å)C. Figure 5 shows that the particle size of both LDL and HDL remained constant upon incubation with up to 0.33% TG(20Å)C (22.0 ± 1.9 and 12.5 ± 0.4 nm, respectively). This indicates that the integrity of the lipoprotein particles is maintained even at concentrations of up to 3.3 mg of TG(20Å)C/mL. Incubation with digitonin, a spirostane-derivatized oligosaccharide with established detergent activity resulted in a dramatic increase of the particle size of HDL and LDL at concentrations as low as 0.01 and 0.0055%, respectively, which is indicative of disintegration of both lipoproteins upon incubation with digitonin. As a result

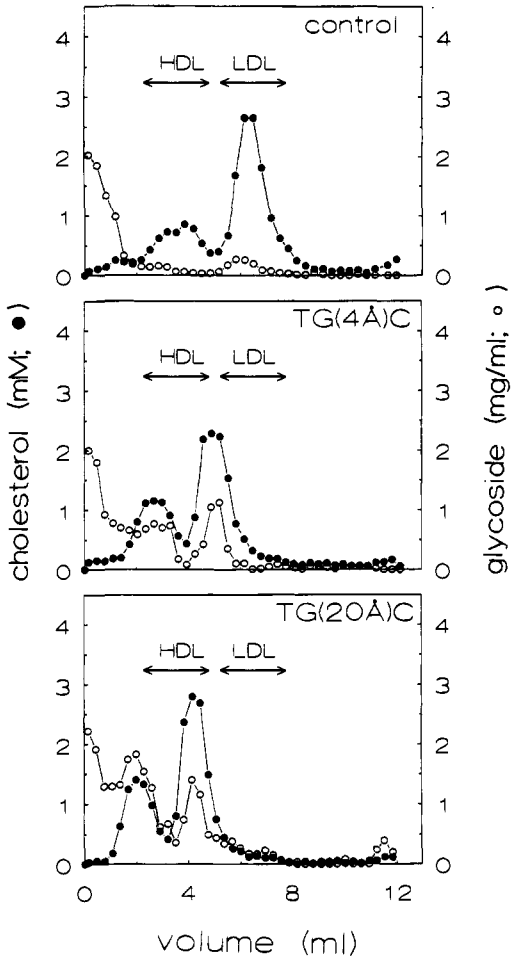

Figure 4. Density-gradient ultracentrifugation of human serum (control), human serum incubated with TG(20Å)C and human serum incubated with TG(4Å)C. Distribution of cholesterol (●) and glycoside (○) over the density-gradient fractions after ultracentrifugation of 1.3 mL of human serum, which has been incubated for 10 min at room temperature with PBS (0.2 mL; top panel) or PBS (0.2 mL), containing 2.2 mg of TG(20Å)C (1.2 μmol; bottom panel) or 1.5 mg of TG(4Å)C (1.2 μmol; middle panel). The amount of total cholesterol content that was recovered from serum and TG(4Å)C- and TG(20Å)-treated serum after density-gradient ultracentrifugation was 6.58, 6.60, and 6.24 μmol (3.69 ± 0.16 μmol of LDL and 1.79 ± 0.12 μmol of HDL), respectively.

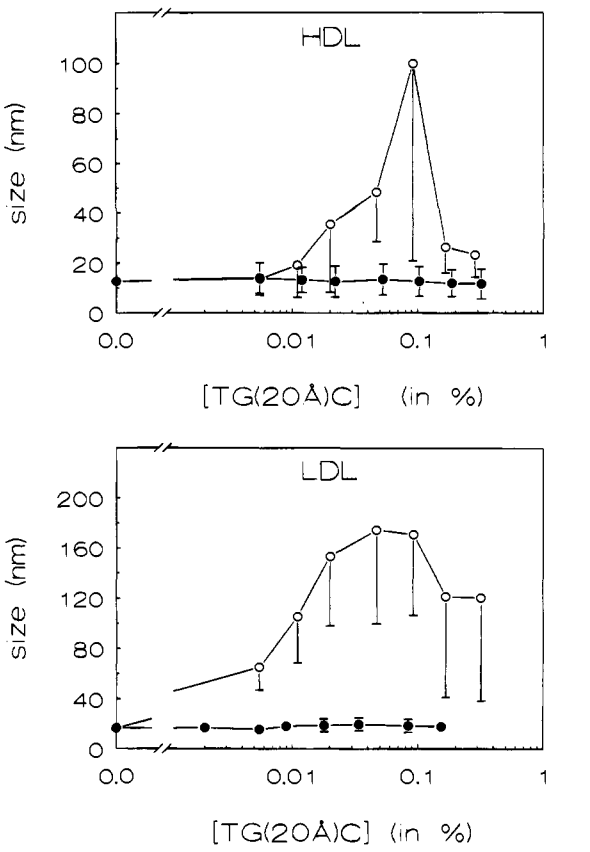

Figure 5. Size of HDL and LDL upon incubation with TG(20Å)C (●) or digitonin (○). HDL (2 mg/mL PBS) and LDL (0.5 mg/ml PBS) were incubated for 10 min at room temperature with 0.0–0.33% TG(20Å)C or digitonin (w/w). After incubation, the particle size of the lipoproteins was scanned by photon correlation spectroscopy at 25 °C. Plotted is the mass-weighed mean of the particle size (nm) ± standard error.

of the very high polydispersity of the lipoprotein/digitonin micelles, the standard error of the size analyses of these mixtures is high.

Biological Activity of the Cholesterylated Cluster Galactoside TG(20Å)C. As published earlier, TG(4Å)C did not display any hypocholesterolemic activity after intravenous bolus injection in rats of up to 25 mg/kg.[12] Continuous infusion of 30–85 mg of TG(4Å)C/kg was required to accomplish a significant reduction of the serum cholesterol level in rats.[12] It can be rationalized that the moderate hypocholesterolemic activity may be linked with the rather low affinity of the galactoside component from TG(4Å)C for the asialoglycoprotein receptor on the parenchymal liver cell. In previous studies we have demonstrated that the affinity of TG(20Å) for the asialoglycoprotein receptor is in the nanomolar range (K_i = 200 nM), which is 2000-fold higher affinity than that of the galactoside component from TG(4Å)C (K_i = 390 μM).[17] Moreover, preliminary studies have revealed that the low affinity of the TG(20Å) for the likewise galactose-recognizing fucose receptor on Kupffer cells[22,23] is not affected by elongation of the spacer from 4 to 20 Å,[24] suggesting that elongation of the spacer also enhances the specificity of the cluster galactoside for the asialoglycoprotein receptor. A recent study has established that LDL and HDL are targeted to the liver upon preincubation with TG(20Å)C.[25] In contrast to TG(4Å)C and mono-Gal-chol, TG(20Å)C-induced liver uptake of both lipoproteins is mediated by the asialoglycoprotein receptor on parenchymal liver cells. Combined with induced lowering of the above results, this indicates that the TG(20Å)C serum cholesterol level results from incorporation of TG(20Å)C into serum lipoproteins and subsequent targeting of these lipoproteins to the liver.

To assess the hypocholesterolemic activity of TG(20Å)C in vivo, we have performed dose–effect studies of intravenously administered TG(20Å)C in rats. The rat may not be the most appropriate animal model in terms of cholesterol metabolism and serum lipoprotein profile. Nonetheless, its use enables a direct comparison of data obtained using TG(20Å)C with previous studies of the physiological effect of TG(4Å)C and mono-Gal-chol 1.[10–15] A dose-dependent decrease of the serum cholesterol level was observed both at 3 h 20 min and at 11 h after intravenous injection of TG(20Å)C (Figure 6). At a dose of 1.9 mg/kg, the level was reduced by 45% ($p < 0.01$), but even at a dose of 0.18 mg/kg, a slight though not significant decrease of the cholesterol level was noticed. In contrast to TG(4Å)C,[12] intravenous

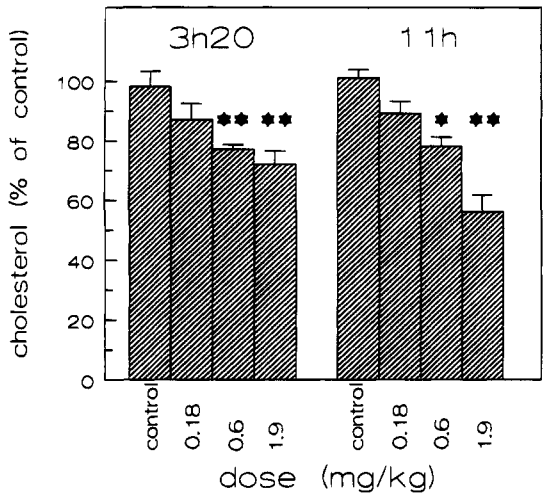

Figure 6. Effect of intravenous injection of TG(20Å)C on the total cholesterol content of rat serum: PBS (500 μL) or PBS containing 0.18, 0.6 and 1.9 mg/kg TG(20Å)C was injected in the vena penis. At 3 h 20 min and at 11 h after injection, blood samples were collected by orbital punction. The blood samples were centrifuged, and the total cholesterol content of the serum was determined in duplicate, using the CHOD-PAP kit of Boehringer Mannhein. The values are means ± sd of three experiments. Significance of the difference between TG(20Å)C-treated rats and the control is indicated by ★★ ($p < 0.01$) and ★ ($p < 0.05$).

bolus injection of at least 6.0 mg/kg TG(20Å)C into rats did not cause any haemolysis and was tolerated well.

In conclusion, we have synthesized the cholesterol derivative of a triantennary cluster galactoside with high affinity for the asialoglycoprotein receptor: TG-(20Å)C. In aqueous solution, TG(20Å)C forms micelles with a size of approximately 4.9 nm rendering it water-soluble. TG(20Å)C associates spontaneously with lipoproteins upon mixing with serum. Incubation of LDL and HDL with up to 0.33% TG(20Å)C (w/v) does not detectably affect the integrity of these lipoproteins. In rats, a single injection of TG(20Å)C led to a significant reduction of the serum cholesterol level. TG(20Å)C appears to exert an hypocholesterolemic activity at a 30-fold lower doses than a previously developed cholesterylated galactoside, TG(4Å)C. This increased efficacy of TG(20Å)C to lower the serum cholesterol levels may arise from the strongly improved affinity of TG(20Å)C for the asialoglycoprotein receptor on the parenchymal liver cell as compared to TG(4Å)C. Recent studies have demonstrated that TG(20Å)C induces hepatic uptake of lipoproteins like LDL and HDL in the rat.[25] For both lipoproteins, the asialoglycoprotein receptor appeared to be responsible for the TG(20Å)C-induced hepatic uptake.[25] This suggests that TG(20Å)C lowers serum cholesterol levels by inducing hepatic uptake of serum lipoproteins, as was also the case for TG(4Å)C-induced reduction in serum cholesterol levels.[12] Further studies on TG(20Å)C-induced cholesterol clearance and the fate of cholesterol(esters) from serum lipoproteins are required to conclusively elucidate the proposed mechanism for TG(20Å)C-induced lowering in serum cholesterol. Eventually, this information will enable us to validate the clinical relevance of TG(20Å)C as a hypolipidemic agent in hypercholesterolemic patients.

Experimental Section

Chemicals and Solvents. (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was purchased from Bissendorf Biochemicals GmBh (Hannover, FRG). Digitonin was obtained from Fluka (Basel, Swiss). *N,N*-Dimethylformamide (DMF), and *N,N*-dimethylacetamide (DMA) were stirred for 16 h with CaH_2 (5 g/L) and then distilled under reduced pressure. Dichloroethane (DCE), dichloromethane (DCM), 1,4-dioxane, and pyridine were refluxed for 16 h with CaH_2 (5 g/L), distilled, and stored over molecular sieves 4 Å, 4 Å, 4 Å, and 5 Å, respectively. Diethyl ether (Et_2O), from Janssen (Beerse, Belgium), 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 and ethanol were dried over magnesium methoxide or ethoxide, respectively, which was prepared in situ by iodine-activated magnesium curls (5 g/L) in methanol/ethanol (Janssen, Beerse, Belgium), refluxed for 1 h, and distilled. Evaporations were carried out under reduced pressure (15 or 0.5 mmHg) at bath temperatures of 20–40 °C. Triethylammonium bicarbonate buffer (TEAB, 2M) was prepared by passing a stream of CO_2 gas through a cooled solution of triethylamine (825 mL) in deionized water (2175 mL) until saturation (pH = 7.0). *N*-[[Tris-*O*-(β-D-galactopyranosyl)-methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)succinyl]glycinamide (TG(4Å)C) was synthesized as described by Kempen et al.[11] The synthesis of *N*-[[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-methyladipyl)]glycinamide (TG(20Å); **2**) is described in detail elsewhere.[17]

Chromatography. Thin layer chromatography was performed using silica F_{254} preformed 0.1 mm thick layers on a plastic backing (Schleicher & Schuell DC- Fertigfolien F1500) in the following mobile phases: A, DCM/MeOH (95/5, v/v); B, DCM/MeOH/NH_4OH (95/5/1, v/v/v); C: DCM/MeOH/NH_4OH (90/10/2, v/v/v), and D, MeOH. Carbohydrates were visualized after spraying with 20% H_2SO_4 in MeOH and subsequent heating to 140–160 °C. Nitrogen-containing compounds (amines and amides) were visualized either using a ninhydrin spray followed by heating to 120–140 °C, or after exposition to Cl_2 vapor for 1 min, and subsequent soaking of the TLC sheet in a solution of *o*-toluidine, prepared according to the method of von Arx et al.[26] ("TDM"). Compounds containing unsaturated bonds were detected under UV (254 nm) or after exposition to I_2. Preparative column chromatography was performed on silica (200–400 mesh ASTM, Merck). Column fractions were analyzed on TLC. Gel exclusion chromatography was performed using sephadex LH20 (Pharmacia, Uppsala, Sweden), suspended in DCM/MeOH (1/1; v/v). FPLC high-resolution gel filtration was done on a sephacryl high-resolution S100 column (S100 HR HiLoad XK26 from Pharmacia, Upssala, Sweden). The eluent was *on-line* monitored on UV_{254} adsorption (Uvicord LKB 2158) or refractive index (Diff. Refractometer LKB 2142).

Instruments and Analysis. Melting points (uncorrected) were determined on a Buchi 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 mode (FT). Chemical shifts are denoted in ppm (δ) relative to tetramethylsilane as internal standard. For ^{13}C-NMR spectra, proton noise decoupling was used. Mass spectra were recorded on a Bioïon-PD-MS mass spectrometer using a nitrocellulose matrix.

Syntheses. Glycine-(5-cholesten-3β-yl ester) Hydrotrifluoroacetate (Figure 1, 1). To a solution of *N*-(*tert*-butyloxycarbonyl) glycine (5 mmol; 890 mg) and *N,N'*-dicyclohexylcarbodiimide (5 mmol, 1.03 g) in dichloroethane (100 mL) was added 3.87 g of cholesterol (10 mmol) and a catalytic amount of (*N,N*-dimethylamino)pyridine (1 mmol, 120 mg), and the solution was stirred for 3 h at 50 °C. The reaction mixture was filtered and extracted with 10% $NaHCO_3$ (2 × 100 mL) and H_2O (2 × 100 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was chromatographed over a Kieselgel-60 column (50 g) using MeOH:DCM = 1:5 as eluent. Crystallization of crude the crude product from Et_2O/H_2O yielded 2.23 g of pure *N*-(*tert*-butyloxycarbonyl)glycine-(5-cholesten-3β-yl ester) (4.1 mmol; 82%): mp 110–112 °C; R_f = 0.76 (system C; TDM); ^1H NMR ($CDCl_3$) δ 5.39 (d, 1H, H6-chol), 5.19 (t, J = 3 Hz, 1H, NH), 4.67 (m, 1H, chol-3H), 3.83 (d, J = 3 Hz, 2H, CH_2-Gly), 2.39–

0.7 (m, 51H, chol), 1.49 (s, 3H, CH_3-*tert*-butyl). Deprotection of the *tert*-butyloxycarbonyl group of *N*-(*tert*-butyloxycarbonyl)-glycine-(5-cholesten-3β-yl ester) was accomplished by treatment with trifluoracetic acid (TFA) as follows. *N*-(*tert*-Butyloxycarbonyl)glycine-(5-cholesten-3β-yl ester) (1 mmol, 0.55 g) was dissolved in DCM:TFA (10 mL; 4:1) and stirred for 30 min at room temperature. To remove residual TFA, the mixture was repeatedly diluted with toluene and concentrated under reduced pressure. Crude **1** was crystallized from EtOH/DCM as a trifluoracetate salt, yielding 520 mg of a white solid (0.92 mmol, 92%; **1**): mp 182 °C dec; R_f = 0.50 (B; Ninh); ^{1}H NMR ($CDCl_3$) δ 5.14 (m, 1H, H6-chol), 4.40 (m, 1H, H3-chol), 3.72 (s, 2H, CH_2-Gly), 0.71–2.46 ppm (m, 42H, chol).

***N*-[[Tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl-N^{α}-(1-adipyl)glycinamide (Figure 1, 3).** Compound **2** (900 mg, 0.6 mmol; Figure 1) was dissolved in 1,4-dioxane:H_2O (77:23, 9.75 mL), and NaOH (4 M, 0.25 mL) was added.[18] The solution was stirred for 15 min at 20 °C, after which the reaction mixture was neutralized by addition of acetic acid. The solvent was removed under reduced pressure, and the crude product was subsequently applied to a Sephacryl S100-HiLoad column using 100 mM TEAB as eluent. The front peak fractions were combined, concentrated, and lyophilized to yield 340 mg of product **3** (0.24 mmol, 72%). TLC analysis revealed a single spot at R_f = 0.23 (D; H_2SO_4): mass (PD; M + Na^+) 1434.1 (M_{calc} = 1434.5). Anal. ($C_{57}H_{105}N_2O_{37}$·$8H_2O$ triethylammonium salt) C, H, N.

***N*-[[Tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methoxymethyl]methyl]-N^{α}-[1-(6-(5-cholesten-3β-yloxy)glycyl)adipyl]glycinamide (Figure 2, 4; TG(20Å)C).** To a stirred solution of compound **3** (340 mg, 0.24 mmol), diisopropylethylamine (0.5 mmol, 80 μL), and **1** (0.5 mmol, 230 mg) in DMA (7 mL) was added (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (0.25 mmol, 100 mg). After 30 min at 20 °C, the reaction mixture was concentrated, and the residue was dissolved in water and subsequently lyophilized. The crude product was subjected to size-exclusion chromatography over LH20 (methanol as eluent), followed by chromatography over Kieselgel 60 (MeOH as eluent) to yield 201 mg of homogeneous TG(20Å)C (0.11 mmol, 46%): R_f (**4**)=0.38, R_f (**3**)=0.23 (D, H_2SO_4); mp 220 °C dec; mass (PD, M + Na^+) 1858.7 (M_{calc} = 1859.6; Figure 3); Anal. ($C_{86}H_{153}N_3O_{38}$·$6H_2O$) C, H, N. ^{1}H NMR (D_2O) δ 5.21 (m, 1H, H6-chol), 4.77 (s, broad, OH-Gal), 4.43 (m, 1H, H3-chol), 4.17 (d, J = 7.5 Hz, 3H, H1-Gal, β-configuration), 3.99–3.80 (m, 6H, C-CH_2), 3.76 (m, 5H, CH_2-Gly and H4-Gal), 3.64 (s, 42H, *O*-CH_2 from tetra(ethylene glycol)), 3.56 (m, 6H, H6-Gal), 3.31 (m, 9H, H2,3,5-Gal), 2.40 (m, 2H, H4-chol), 2.31 (t, 2H, H2-adipyl), 1.62 (q, 4H, H3,4-adipyl), 2.40–0.87 (m, 48 H, chol and H5-adipyl); ^{13}C{^{1}H}-NMR (D_2O). δ 177.0–171.1 (4 × C=O amides, ester), 139.2 (C5-chol), 122.0 (C6-chol), 103.7 (C1-Gal, β-configuration), 96.2 (methyl acetal, O–C–O), 75.9 (C5-Gal), 73.5 (C3-Gal), 73.4 (C3-chol), 71.7 (C2-Gal), 70.5 (C5-chol), 70.2–70.3 (C's from tetra(ethylene glycol)), 70.0 (C4-Gal), 69.4 (C6-Gal), 67.4 (C's from tris(hydroxymethyl)), 61.7 (quaternary C), 56.3 (C14-chol), 52.8 (O-CH_3), 43.5 (Cα-Gly), 37.2 (C2-adipyl), 35.8 (C5-adipyl), 34.1 (C-chol), 28.7 (C-chol), 25.7 (C3-adipyl), 24.5 (C4-adipyl), 23.5–11.7 (remaining C atoms from cholesterol).

Association of TG(20Å)C with Human Serum Constituents. Association of TG(20Å)C (**4**) and TG(4Å)C (**5**) with serum lipoproteins from human serum was determined as follows. Human serum (1.3 mL) was incubated for 10 min at room temperature with 0.2 mL of phosphate-buffered saline (NaP_i, 10 mM; NaCl, 150 mM, pH=7.40; PBS) or PBS, containing TG(20Å)C (2.2 mg) or TG(4Å)C (1.5 mg). Subsequently, the mixtures were subjected to density gradient ultracentrifugation in KBr/NaCl solutions according to the procedure of Redgrave et al.[27] Following centrifugation, the gradient is subdivided into 0.35 mL fractions, and the fractions were analyzed for cholesterol content using the CHOD-PAP kit (Boerhinger Mannheim) and for carbohydrate content according to the procedure of Spiro.[28]

Particle Size Analysis. The micellar particle size of TG(20Å)C, TG(4Å)C, and digitonin (a spirostane-derivatized oligosaccharide), dissolved in PBS at a concentration of 5 mg/mL, was determined by photon correlation spectroscopy.[29–31] Photon correlation spectroscopy is based on dynamic laser light scattering and yields a very good estimation of the average size of even heterodispersic particles at sizes ranging from 5 nm to 5 μm. For analysis of the particle size of lipoproteins and lipoprotein/glycolipid mixtures, this technique was preferred to the conventional method using native gelelectrophoresis, since it is less sensitive to particle charge, particle dimensions, and interaction of particles with the polyacrylamide matrix. Moreover, it allows measurement of the particle size in any buffer of choice. The average particle size of the lipoprotein and lipoprotein/glycolipid containing solutions were monitored on a Malvern 4700C laser light scattering apparatus (equipped with an Malvern PCS100 spectrometer and a 7032 multi-8 computing correlator) at 26.0 °C, a detection angle of 120°, and 632 nm for an accumulation time of 72 s. The spectroscopic data (% in range >95%) were analyzed according to the Raleigh–Gans–Debeye theory to evaluate the apparent mass-weighed particle-size distribution.[32]

Isolation of Lipoproteins from Human Serum. Low-density lipoprotein (LDL) ($1.019 < d < 1.063 \times 10^3$ kg/m^3) and high-density lipoprotein (HDL) ($1.063 < d < 1.21 \times 10^3$ kg/m^3) were isolated from human serum by gradient density ultracentrifugation in KBr/NaCl solutions according to the procedure of Redgrave et al.[27]

Interaction of TG(20Å)C with HDL and LDL. The integrity of human LDL (0.5 mg/mL) and HDL (2.0 mg/mL) after incubation for 10 min at room temperature with TG(20Å)C (0–0.33% w/v) in PBS was determined by monitoring the mass-weighed particle size distribution of the lipoprotein/galactoside mixtures as described above. As a control for disintegration of the tested lipoproteins, the detergent digitonin (a spirostane derivatized oligosaccharide) was incubated with LDL and HDL at a concentration ranging from 0 to 0.33% (w/v), and the particle size was determined as described above.

Animal Experiments. Rats (male, Wistar, 250–300 g) were anaesthetized with ether, and 300 μL blood samples were collected by orbital puncture. TG(20Å)C (0.18, 0.6, or 1.9 mg/kg in 500 μL of PBS) or 500 μL of PBS was injected iv in the vena penis, and blood samples were taken by orbital puncture at 3 h 20 min and 11 h after injection. After sampling, the blood samples were centrifuged for 5 min at 1500*g*, and the serum was collected and stored at 4 °C for further analysis. The total cholesterol content of the serum was determined colorimetrically in triplicate, using the CHOD-PAP kit (Boerhinger Mannheim). TG(20Å)C did not interfere with the cholesterol assay both in the absence or presence of cholesterol esterase.

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