A Water-Soluble Cholesteryl-Containing Trisgalactoside: Synthesis, Properties, and Use in Directing Lipid-Containing Particles to the Liver

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The synthesis of a trisgalactoside-terminated cholesterol derivative is described. Tris(galactosyloxymethyl)-aminomethane is coupled to cholesterol by using glycyl and succinyl as intermediate hydrophilic spacer moieties. The resulting cholesteryl ester dissolves easily in water, forming monodisperse micelles. When added to dispersions of liposomes or plasma lipoproteins in water, the substance becomes incorporated rapidly into these structures, causing an increase of their buoyant density. Liposomes or low-density lipoproteins, preloaded with the substance, are rapidly cleared from the circulation and taken up by the liver after intravenous injection in rats. This uptake is inhibited by *N*-acetylgalactosamine but not by *N*-acetylglucosamine, indicating the specificity of this process.

On their plasma membrane mammalian liver cells possess specific receptors for glycoproteins, whose oligosaccharide chains have galactose or *N*-acetylgalactosamine as their terminating unit.[1-3] Binding of such glycoproteins or synthetic glycoconjugates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain: triantennary structures are bound with greater affinity than biantennary or monoantennary chains.[4,5] This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates.[6,7] Binding at 37 °C is followed by uptake of the glycoprotein–receptor complex in a process called absorptive endocytosis.

Various groups of investigators have achieved an increased hepatic uptake of substances by encapsulating them in liposomes containing galactosyl-terminating glycoconjugates.[8-11] Alternatively, plasma proteins have been chemically modified in vitro by coupling with galactosyl-terminating conjugates, also resulting in an enhanced binding and uptake by liver cells.[12,13] For example, Attie et al.[12] found that lactosylated low-density lipoprotein (LDL) was internalized by rat hepatocytes in primary culture at a much higher rate than native LDL. The latter findings prompted us to construct a synthetic glycoconjugate with cholesterol, having the purported ability to become associated with liposomes or with naturally occurring lipoproteins after its systemic administration, and to enhance thereby the clearance of these particles from the blood by the liver.

Lee and co-workers have synthesized a series of cluster glycosides by glycosylation of 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris).[5,13,14] We have coupled such a Tris–galactoside derivative to cholesterol using glycine and succinic acid as spacer moieties. In this paper we report the synthesis and some properties of the glycopeptidolipid.

Results and Discussion

Synthesis. The synthesis of **5** was initiated by coupling Tris with (benzyloxycarbonyl)glycine to yield the amido compound **2** by use of *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline. This amide was then glycosylated with tetra-*O*-acetyl-α-D-galactosyl bromide (**1**) according to the method of Helferich and Zinner.[15] The fully protected product **3a** was obtained as an apparently homogeneous glass in 32% yield after repeated purification by silica gel column chromatography. The β-configuration of the glycosidic bonds in **3a** was established by ^1H and ^{13}C NMR spectroscopy.

Complete deblocking of the fully protected glycopeptide **3a** was effected in two stages. Firstly, treatment with triethylamine/methanol/water removed the acetyl groups to give **3b**. Subsequently, deblocking of the benzyloxycarbonyl group from **3b** using catalytic hydrogenation (Pd/C) followed by gel filtration on Sephadex G-10 afforded **3c** as the HCl salt in 72% yield (based on **3a**). TLC analyses of **3b** and **3c** indicate each of these products to contain a major and a minor component, which are assigned to the trigalactosylated and digalactosylated derivative, respectively (see below). It was decided to separate the differently glycosylated compounds after the condensation with cholesteryl hydrogen succinate.

Compound **3c** was coupled with the *N*-hydroxysuccinimidyl ester of cholesteryl hydrogen succinate.[16] The advantage of having a glycine moiety in **3c** is that its amino group is considerably more reactive than that of (glycosylated) Tris,[17] minimizing the extent of *O*-acylation of the

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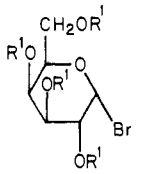
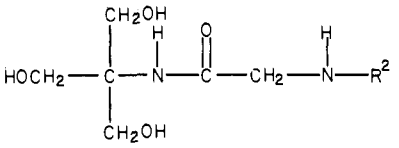


1, R^1 = acetyl



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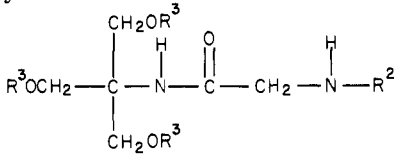
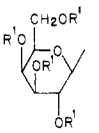
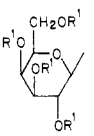
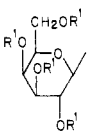
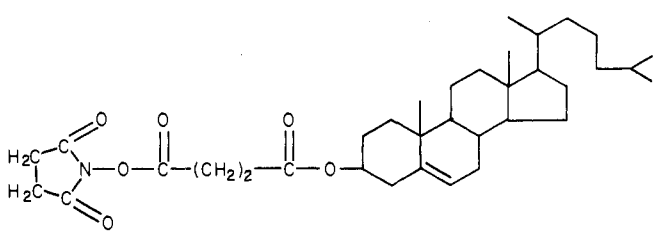


3a, R^1 = acetyl; R^2 = benzyloxycarbonyl; R^3 = 

b, R^1 = H; R^2 = benzyloxycarbonyl; R^3 = 

c, R^1 = H; R^2 = H; R^3 = 

galactosyl moieties. A solution of **3c** in DMF/DMA was reacted with the activated ester **4** in the presence of NEM.



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Coupling was complete after 3.5 h at room temperature. After workup the product was found to contain a major and a minor component, which could fully be separated by column chromatography on silica (eluant: chloroform/methanol/water, 54/40/6) with yields of 50% and 10%, respectively (based on **3c**). On the basis of ^{1}H NMR spectra, elemental analysis, and the molar proportion of assayable cholesterol and galactose after alkaline or acid hydrolysis, the major coupling product was identified as the trisgalactoside **5** (see Experimental Section). The minor component was identified as the bisgalactoside **6** by using ^{1}H NMR.

Interactions of 5 with Dispersions of Lipid in Water. Compound **5** is readily soluble in water, in which it forms micelles since it is not dialyzable and is quantitatively recovered from the content of the dialysis tube. Upon chromatography on Sepharose CL-6B an apparent molecular weight (M_r) of about 4×10^4 is found for **5** (Figure 1), indicating the presence of about 35 molecules per micelle. Although cholesteryl-containing glycolipids have been synthesized by others before,[18,19] these were not water soluble and their incorporation into liposomes (uni- or multilamellar vesicles) required the presence of the glycolipid in the lipid mixture subjected to sonication.

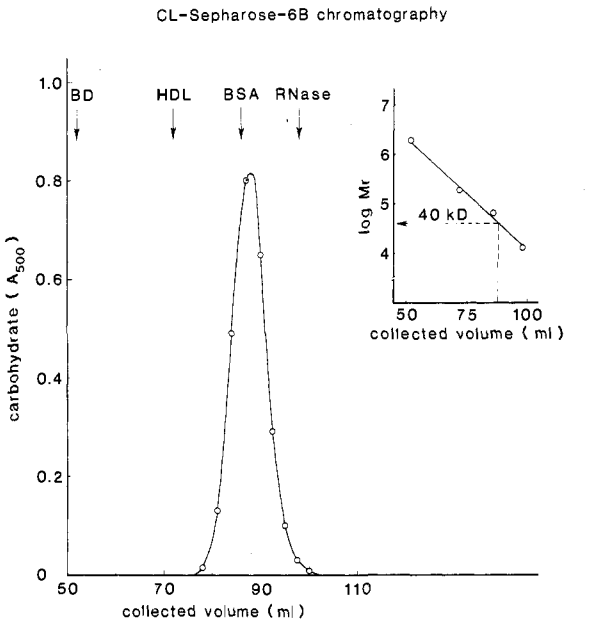

Figure 1. Chromatography of glycolipid **5** on Sepharose-CL-6B. The column (80 × 1.6 cm) was loaded with 7 mg of glycolipid **5** dissolved in 2 mL of 10 mM NH_4HCO_3 buffer and eluted using the same buffer of at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected and assayed for carbohydrate (phenol–sulfuric acid reaction). The arrows indicate the elution peaks of the following substances chromatographed in the same conditions: Blue dextran (BD), M_r 2.10^6; high-density lipoprotein isolated from human plasma (HDL), M_r 3.10^5; bovine serum albumin (BSA), M_r 69.10^3; RNAse A, M_r 14.10^3). Insert: plot of log M_r vs. elution peak of the various substances, indicating a M_r of 4.10^4 for glycolipid **5** in these conditions.

Since compound **5** is water soluble, it was of interest to see whether it could become incorporated in preformed liposomes by direct addition of **5** to the liposomes suspension in water.

As shown in Figure 2A, liposomes prepared from sphingomyelin/cholesterol (1/1) are found to equilibrate at density peaks around 1.035 and 1.08 when subjected to density gradient ultracentrifugation in KBr/NaCl solutions.[20] On the other hand, compound **5** is completely sedimented to the bottom of the tube under these conditions (Figure 2B). When compound **5** is added to a liposome dispersion in water in an amount corresponding to 10 mol % of the liposome–lipids, the liposomes are now found to equilibrate at higher densities in the salt gradient (Figure 2A) and all of compound **5** is found in the same density fractions as the liposomes (Figure 2B). This indicates that **5** has become associated with the liposomes in their aqueous environment.

The liposomes can be prepared with a water-soluble radioactive substance enclosed in their internal water space. The leakage of [^{3}H]inuline from these vesicles after density gradient ultracentrifugation amounts to 22.8% and is decreased to 9.5% or 11.2% by addition of 10 mol % or 20 mol %, respectively, of **5** to the vesicles. This finding is consistent with the data reported by Orr et al.,[19] who incorporated a monoglycosylated cholesterol derivative into liposomes.

The association of **5** with lipid dispersions in water could also be observed when the compound was dissolved in human citrated plasma. The distribution of cholesterol among density fractions, after ultracentrifugation in the way described above, is shown in Figure 3A. When ^{3}H-

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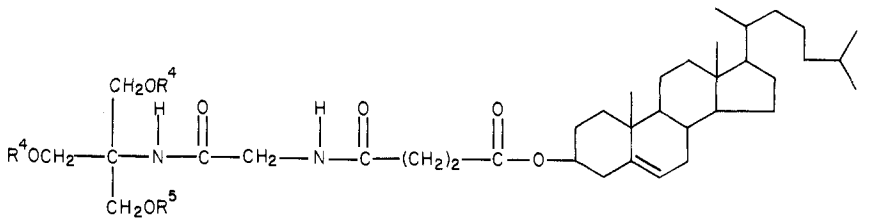
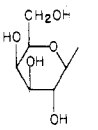


5, $R^5 = R^4 =$ 

6, $R^5 = H$

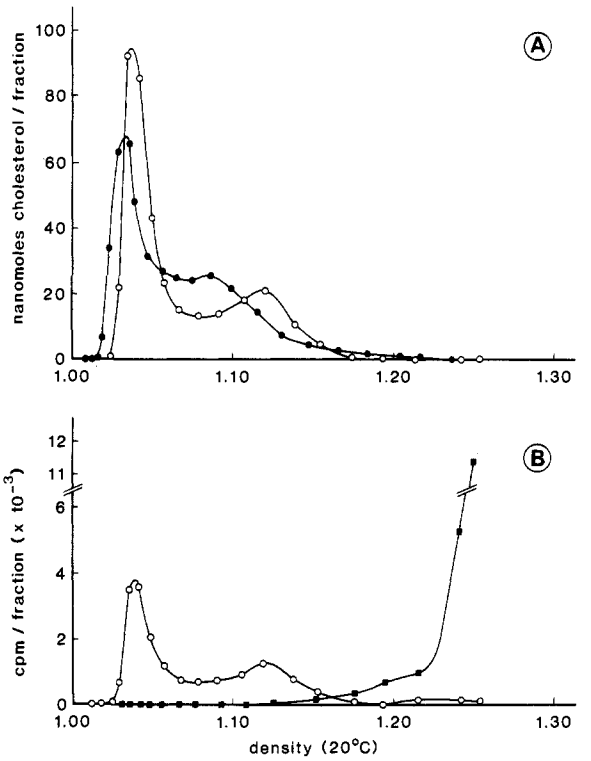


Figure 2. Density gradient ultracentrifugation of liposomes, of glycolipid **5**, or of liposomes incubated with the glycolipid. Upper panel (A): distribution of free cholesterol after centrifugation of liposomes alone (4.0 μmol of total lipid) (●) or of liposomes (3.6 μmol of total lipid) incubated with 0.4 μmol of ^{3}H-labeled glycolipid **5** (○). Lower panel (B): distribution of ^{3}H radioactivity among the fractions after centrifugation of ^{3}H-labeled glycolipid **5** alone (0.4 μmol) (■) or of liposomes (3.6 μmol of total lipid) incubated with 0.4 μmol of ^{3}H-labeled glycolipid **5** (○).

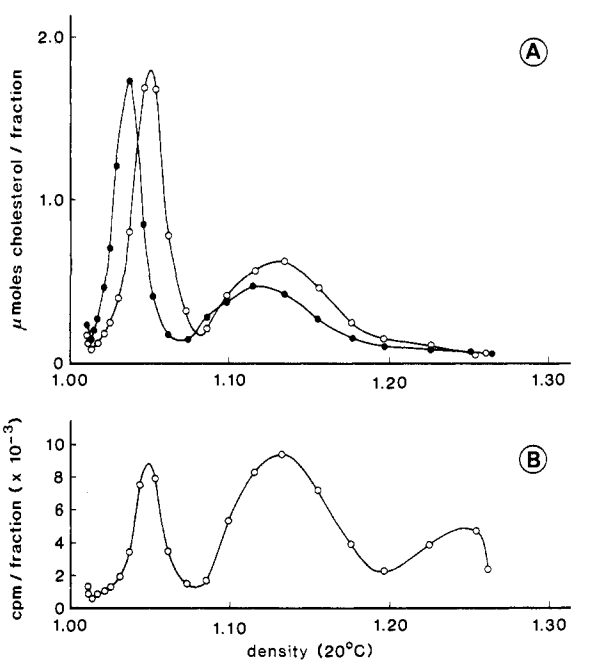


Figure 3. Density gradient ultracentrifugation of human plasma or of plasma incubated with glycolipid **5**. Upper panel (A): distribution of cholesterol (free plus esterified) among the fractions after centrifugation of 1.8 mL of human plasma alone (●) or of 1.8 mL of human plasma mixed with 0.2 mL of saline containing 1.7 μmol of ^{3}H-labeled glycolipid **5** (○). Lower panel (B): distribution of ^{3}H radioactivity among the fractions after centrifugation of the plasma mixed with glycolipid solution.

labeled **5** is added to human plasma to a final concentration of 0.84 mM and the plasma is ultracentrifuged, the peak densities of low-density (LD) and high-density (HD) lipoproteins are shifted to higher values (Figure 3A). As evident from the ^{3}H distribution (Figure 3B), the added **5** is mainly associated with LD and HD lipoproteins, while a minor part is found in the fractions with a density between 1.21 and 1.25.

If it is assumed that a LD particle contains 1800 cholesterol molecules (free and esterified) and a HD particle 45,[21] it can be calculated from the data given in Figure 3 that there are on average 180 molecules of **5** associated with each LD particle and 14 molecules of **5** with each HDL particle. Since the diameter of the LD particle is about 2.5-fold greater than that of the HD particles and the latter contain relatively much more protein per particle, these findings indicate that **5** is distributed among the LD and HD particles roughly in proportion to their exposed lipidic surface area.

When the LD or HD lipoproteins, loaded with ^{3}H-labeled **5**, are mixed with fresh plasma and centrifuged in the same manner again, the label becomes distributed among the density fractions in the same fashion as depicted in Figure 3B. Apparently, substance **5** can readily interchange between the different lipoproteins and (one or more of) the plasma proteins in the $d > 1.21$ fraction.

Preliminary experiments were done to establish the effect of loading liposomes or lipoproteins with **5** on their fate after intravenous injection in rats. As shown in Table I, the rates at which [^{14}C]sucrose-containing liposomes or ^{125}I-labeled LDL are cleared from the blood and taken up by the liver are strikingly increased by preloading these particles with **5**. For LDL we find that the size of the effect clearly depends on the degree of loading. The increase in liver uptake can be blocked nearly completely by pretreating the rats with 110 mg of 2-acetamido-2-deoxygalactopyranoside (iv injected) 1 min before the injection of the labeled preloaded LDL, while preinjection of 110

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Table I. Effect of Association of [^{14}C]Sucrose-Containing Liposomes and [^{125}I]LDL with Compound **5** on Removal of Radioactivity from Blood and on Uptake in the Liver

A. [^{14}C]Sucrose-Containing Liposomes (Sphingomyelin/Cholesterol, 1/1)

loading of liposomes (mol of **5**/ mol of lipid)	^{14}C remaining in blood (120 min after injection)	^{14}C in liver (after perfusion)
0	82 ± 9[a]	3 ± 1
0.1	48 ± 6	48 ± 4

B. [^{125}I]LDL

loading of LDL (nmol of **5**/ μg of LDL-protein)	preinjection	^{125}I remaining in blood (after 10 min)	^{125}I in liver (after perfusion)
0		97 ± 1	1.9 ± 0.2
0.05		96 ± 3	2.0 ± 0.3
0.25		57 ± 10	33 ± 1
0.65		23 ± 9	41 ± 2
0.65	Gal-N-Ac (110 mg)	94 ± 3	3.2 ± 0.5
0.65	Glc-N-Ac (110 mg)	27 ± 3	39 ± 4

[a] Data represent percent of injected radioactivity and are means ± SD for three independent observations with each degree of loading.

mg of 2-acetamido-2-deoxyglucopyranoside had no effect (Table I). This indicates that a galactose-specific recognition site in the liver is responsible for the increased liver uptake. More detailed information on the fate of lipoproteins and liposomes, loaded with **5**, upon injection in living mammals or upon incubation with isolated liver cells, will be published elsewhere. The findings presented here suggest that one can cause the removal of lipoproteins from the plasma by the liver simply by intravenous administration of **5**. Upon testing this we found only a small decrease of the total plasma cholesterol level in rats and rabbits, 1–3 h after injection of up to 30 mg/kg of body weight. Apparently, the degree of occupation of the lipoproteins with **5** rapidly falls below the minimum needed for enhanced removal by the liver. A substance similar to **5** that would remain attached more firmly to lipoproteins (preferably to LDL) after its injection would be a promising hypocholesterolemic agent. Furthermore, the rapid uptake of liposomes or lipoproteins preloaded with **5** by the liver offers a possibility to direct pharmaca toward this organ by attaching them on (or enclosing them within) these particles. This could be an approach when exposure of other organs to these pharmaca is to be minimalized.

Experimental Section

Chemicals and Solvents. Penta-*O*-acetyl-β-D-galactopyranose (PAGP), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), *N*-hydroxysuccinimide (HONSuc), succinic anhydride, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), mercury(II) cyanide, and mercury(II) bromide were obtained from Aldrich, cholesteryl hydrogen succinate, dimyristoyl L-α-phosphatidylcholine, and L-α-phosphatidyl-L-serine from Sigma, acetic anhydride (Ac_2O) and phenol (pa) from Baker, phosphorous tribromide (PBr_3), cholesterol (pa), and acetic acid (pa) (HOAc) from Merck, *N*,*N*-dicyclohexylcarbodiimide (DCCI), triethylamine (TEA) and *N*-ethylmorpholine (NEM) from Fluka, and [1,2-$^3H_2$]cholesterol and [^{3}H]inuline from The Radiochemical Centre. Acetonitrile (CH_3CN) was distilled from P_2O_5; diethyl ether was dried with KOH and distilled; *N*,*N*-dimethylacetamide (DMA) and *N*,*N*-dimethylformamide (DMF) were dried with 4-Å molecular sieves and distilled under reduced pressure; ethanol was dissolved from magnesium ethoxide; pyridine was dried on calcium hydride and distilled from *p*-toluenesulfonyl chloride (50 g/L); tetrahydrofuran (THF) was boiled (6 h) with calcium hydride and distilled. The solvents were stored over 4-Å molecular sieves. Chloroform ($CHCl_3$) was distilled prior to use. Evaporations were carried out under reduced pressure (15 or 0.5 mmHg) at bath temperatures below 40 °C.

Chromatography. Thin-layer chromatography (TLC) was carried out using silica F_{254} preformed layers 0.1-mm thick on a plastic backing (Schleicher and Schüll) in the following mobile phases: A, $CHCl_3$/acetone, 9/1; B, $CHCl_3$/acetone, 8/2; C, $CHCl_3$/methanol, 9/1; D, $CHCl_3$/methanol/water/25% NH_3, 75/25/3/1; E, $CHCl_3$/methanol/water, 54/40/6; F, 1-butanol/HOAc/water, 4/1/1; G, 1-butanol/HOAc/pyridine/water, 15/3/10/12. Spots were visualized either under UV (356 or 254 nm) light or by using one of the following spray stainings: 20% H_2SO_4 in methanol followed by heating to 140–170 °C ("H_2SO_4"); 1% $KMnO_4$ + 1% K_2CO_3 ("$KMnO_4$"); ninhydrin spray (Merck) followed by heating to 110 °C ("Nh").

For preparative column chromatography silica was used with particle sizes "40–63 μm" or "63 μm and smaller" (Merck). The column fractions were analyzed by TLC. For gel filtration Sephadex G-10 or G-50 were used (Pharmacia). Column fractions of these runs were analyzed for carbohydrate by using the phenol–sulfuric acid method.[22]

Instruments and Analyses. Melting points were determined with an apparatus according to Tottoli and are uncorrected. Elemental analyses were done for C, H, and N in compounds **2**, **3a**, **3c**, **4**, and **5**. Additionally, the H_2O content was determined (Fischer method) in compounds **3c** and **5**. UV spectra were measured at room temperature with a Varian–Cary 219 spectrometer. Optical rotations were determined at room temperature with a Perkin-Elmer 141 polarimeter. ^{1}H NMR spectra were measured at 100 MHz with a JEOL JNMPS-100 spectrometer or at 300 MHz with a Bruker WM-300 spectrometer operating in the Fourier-transform (FT) mode. Chemical shifts are given in ppm (δ) relative to tetramethylsilane (Me_4Si) as internal standard. ^{13}C NMR spectra were measured at 25.15 MHz with a JEOL JNMFT-100 spectrometer or at 75.45 MHz with a Bruker WM-300 spectrometer, both operating in the FT mode; proton noise decoupling was used. ^{13}C chemical shifts are given in ppm (δ) relative to Me_4Si as internal standard. For the NMR measurements 10 mM solutions of the substances in the solvent indicated were used.

Syntheses. *N*-(Benzyloxycarbonyl)glycine was prepared as described.[23]

[1α,2α-$^3H_2$]Cholesteryl hydrogen succinate was prepared by using the method of Klein et al.[24] Typically, to a solution of cholesterol (387 mg, 1 mmol) in 10 mL of pyridine was added 60 μCi [1α,2α-$^3H_2$]-cholesterol and 100.3 mg (1 mmol) of succinic anhydride. The solution was heated for 46 h under reflux; the course of the reaction was followed by TLC (solvent D) and counting of the ^{3}H activity in cholesterol (front) and in cholesteryl hydrogen succinate (R_f (D) 0.30). After 46 h, 65% of the radioactivity was present in the form of the ester. The mixture was evaporated to dryness, the residue dissolved in 5 mL of warm acetone, and the solution stored at −20 °C overnight. The mother liquor was removed by careful aspiration, and the crystals were dissolved again in warm acetone and recrystallized by cooling to −20 °C. The product was further purified by column chromatography on silica (25 × 1 cm, 63–250 μm silica particles) using mobile phase D as eluent. Fractions containing solely the ester were pooled and evaporated. The product was not soluble in warm acetone, indicating that the corresponding ammonium salt had been obtained. The free acid was prepared by dissolving the residue in 15 mL of chloroform and washing twice with 10 mL of 0.1 M HCl. The chloroform layer was evaporated under nitrogen. Yield: 300 mg (0.616 mmol, 61.6%) of a homogeneous product, R_f (D) 0.30.

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl Bromide (1). To a stirred and cooled (ice–water bath) solution of penta-*O*-acetyl-β-D-galactopyranose (25 g, 64 mmol) and PBr_3 (38.4 mL)

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in Ac_2O (128 mL) was added dropwise water (54 mL) during a period of 90 min. TLC (solvent A) showed complete conversion. Chloroform (450 mL) was added, and the mixture was extracted with cold water (4 × 500 mL), cold 7% $NaCHO_3$ solution (2 × 500 mL), and cold water (2 × 500 mL). After drying on $MgSO_4$, the chloroform layer was evaporated to dryness. The residue was crystallized in diisopropyl ether/hexane: yield 17.9 g (43.5 mmol, 68%); mp 77–78 °C (lit.[25] mp 79–81 °C); homogeneous on TLC, R_f (A) 0.60 (H_2SO_4); ^1H NMR (100 MHz, $CDCl_3$) δ 6.69 (d, H-1, $J_{1,2}$ = 3.8 Hz, α-configuration), 5.50 (dd, H-4, $J_{3,4}$ = 3.0 Hz, $J_{4,5}$ = 1.5 Hz), 5.40 (dd, H-3, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.4 Hz), 4.99 (dd, H-2, $J_{2,3}$ = 10.5 Hz, $J_{1,2}$ = 3.9 Hz), 4.47 (t, H-5, $J_{5,6A}$ = $J_{5,6B}$ = 6.4 Hz), 4.11 (m, H-6A, H-6B), 2.12 (s), 2.07 (s), 2.05 (s), 1.98 (s) (4 × acetyl CH_3).

N-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]-N$^\alpha$-(benzyloxycarbonyl)glycinamide (2). A solution of Tris (6.1 g, 50 mmol), *N*-(benzyloxycarbonyl)glycine (119.5 g, 55 mmol), and EEDQ (14.8 g, 60 mmol) in absolute ethanol (500 mL) was boiled under reflux for 6 h. The mixture was cooled and the solvent removed by distillation under reduced pressure. The syrup was triturated with diethyl ether (250 mL) and the resulting precipitate collected by filtration, washed with diethyl ether, dried, and dissolved in 100 mL of methanol. Dowex 50 × 4 (H-form) was added until the pH was neutral, The resin was filtered off, the solution was reduced in volume by evaporation, and crystallization of **2** was induced by addition of diethyl ether. **2**: yield 7.5 g (24 mmol, 48%); mp 130 °C; homogeneous on TLC, R_f (C) 0.16 (UV, $KMnO_4$); ^1H NMR (300 MHz, Me_2SO-d_6) δ 7.46 (t, Gly NH, 0.81 (1) H, ^3J = 5.9 Hz), 7.35 (s, C_6H_5, 4.7 (5) H), 7,15 (s, amide NH, 1.0 (1) H), 5.04 (urethane CH_2, 2.1 (2) H), 4.72 (t, OH, 3.0 (3) H, ^3J = 5.7 Hz), 3.65 (d, Gly CH_2, 2.1 (2) H, ^3J = 6.0 Hz), 3.53 (d, C (quat) CH_2, 6.4 (6) H, ^3J = 5.7 Hz); after addition of D_2O the signals at 7.46, 7.15, and 4.72 ppm disappeared and the doublets at 3.53 and 3.65 ppm changed into singlets; ^{13}C NMR (Me_2SO-d_6) δ 169.90 (amide C=O), 156.46 (urethane C=O), 137.04, 128.37, 127.79, and 127.67 (phenyl ring), 65.50 (urethane CH_2), 62.12 (C (quat)), 60.30 (CH_2OH), 43.97 (Gly CH_2); IR (KBr) 1724 (urethane C=O), 1640 (amide C=O), 1555 (amide NHC=O), 1530 cm^{-1} (urethane NHC=O). Anal. ($C_{14}H_{20}O_6N_2$) C, H, N.

N-[Tris[[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)oxy]methyl]methyl]-N$^\alpha$-(benzyloxycarbonyl)glycinamide (3a). A mixture of **2** (3.1 g, 10 mmol), $Hg(CN)_2$ (3.8 g, 15 mmol), and $HgBr_2$ (5.4 g, 15 mmol) in CH_3CN (100 mL) was stirred at room temperature. To this suspension a solution of **1** (12.3 g, 30 mmol) in CH_3CN (100 mL) was added dropwise during 30 min. The reaction mixture gradually cleared during this addition. After 90-min reaction time at room temperature, additional portions of **1** (4.1 g, 10 mmol), $Hg(CN)_2$ (5 mmol), and $HgBr_2$ (5 mmol) were added, and the reaction was left overnight. The mixture was evaporated to dryness, and the residue was suspended in $CHCl_3$ (350 mL). This suspension was washed with 1 M KBr (5 × 125 mL) and water (5 × 120 mL). The $CHCl_3$ extract was dried by using $MgSO_4$ and evaporated to dryness. According to TLC, the resulting crude mixture contained a main product tentatively identified as **3a** (R_f (B) 0.20, H_2SO_4), and a number of side products (R_f (B) 0.03, 0.22, 0.25, 0.31, 0.37, and 0.42; H_2SO_4). The mixture (11 g) was purified by column chromatography on silica (11.7 × 7.8 cm; 230 g of silica particles of 63 μm and smaller) eluting with $CHCl_3$/acetone (8/2). Fractions containing the main product were pooled and evaporated to dryness, yielding 7.2 g of solid residue. This product was further purified in four portions on a column of silica (16 × 4.3 cm; 110 g of silica particles of 63 μm and smaller) eluting with $CHCl_3$/acetone (82/18). Fractions containing the main product and minimally contaminated with side products were pooled and evaporated to dryness. **3a**: total yield 3.1 g (2.37 mmol, 23.7%); mp 93–95 °C; TLC analysis showed the substance to be homogeneous (R_f (B) 0.20; H_2SO_4) or nearly homogeneous (side product R_f (B) 0.22; H_2SO_4); ^1H NMR (300 MHz, $CDCl_3$) δ 7.36 (s, C_6H_5, 4.8 (5) H), 6.17 (s, amide NH, 0.8 (1) H), 5.60 (br s, Gly NH, 0.8 (1) H), 5.39 (br d, H-4, 3.0 (3) H, $J_{3,4}$ = 2.6 Hz), 5.15[a] (s, urethane CH_2) and 5.13[b] (dd, H-2, $J_{2,3}$ = 10.6 Hz, $J_{1,2}$ = 7.6 Hz), a + b (4.9 (5) H), 5.02 (dd, H-3, 3.1 (3) H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 3.3 Hz), 4.41 (d, H-1, 2.8 (3) H, $J_{1,2}$ = 7.7 Hz, β-configuration), 4.18[c] (m) and 4.13[c] (m, H-6A and H-6B, $J_{6A,6B}$ = 11.1 Hz, $J_{5,6A}$ = 6.9 Hz, $J_{5,6B}$ = 5.6 Hz), and ca. 4.12[d] (d, C (quat) CH_2, ^2J = 9.8 Hz), c + d (8.7 (9) H), 3.92 (t, H-5, 3.1 (3) H, $J_{5,6A}$ + $J_{5,6B}$ = 13.5 Hz), 3.82[e] (m, Gly CH_2) and 3.78[f] (d, C (quat) CH_2, ^2J = 10.4 Hz), e + f (4.4 (5) H), 2.15 (s), 2.074 (s), 2.072 (s), 9 (s) (4 acetyl CH_3, 35 (36) H); ^{13}C NMR ($CDCl_3$) δ 170.24, 170.02, 169.81, 169.35 (4 acetyl C=O), 168.78 (Gly C=O), 156.25 (urethane C=O), 136.30, 128.38, 127.95, and 127.77 (phenyl ring), 101.23 (C-1, β-configuration), 70.71 (C-3 or C-5), 70.41 (C-5 or C-3), 68.92 (C-2), 68.07 (C (quat) CH_2), 66.83 (C-4 and urethane CH_2), 61.00 (C-6), 59.03 (C (quat)), 44.38 (Gly CH_2), 20.69, 20.57, and 20.51 (4 × acetyl CH_3). Anal. ($C_{56}H_{74}O_{33}N_2$) C, H, N.

N-[Tris[(β-D-galactopyranosyloxy)methyl]methyl]glycine Hydrochloride Salt (3c). Compound **3a** was deacetylated by adding water (25 mL) and TEA (3.43 mL, 24.6 mmol) to a solution of **3a** (2.68 g, 2.05 mmol) in methanol (25 mL). The solution was stirred for 120 min at room temperature and acidified with HOAc (1.40 mL, 24.5 mmol). The mixture containing **3b** was evaporated to dryness by distillation under reduced pressure and lyophilization. TLC shows a major component (R_f (F) 0.15; R_f (G) 0.43; H_2SO_4) and a minor component (R_f (F) 0.31; R_f (G) 0.51). Attempts to remove the TEA–HOAc salt in the product either by extraction with $CHCl_3$ and diethyl ether or by lyophilization were unsuccessful. For removal of the protective *N*-benzyloxycarbonyl group in **3b**, the lyophilized material (2.05 mmol) was dissolved in HOAc/water (6/4, 100 mL). After flushing with nitrogen gas, 10% Pd/C (0.9 g) was added together with additional solvent (25 mL). Hydrogen gas was then led through the solution at atmospheric pressure for 7 h at room temperature. The reduction appeared to be complete upon TLC analysis (solvent F or G). The catalyst was removed by filtration and the filtrate lyophilized. In order to remove the TEA–HOAc salt remaining from the deacetylation step, the product was purified in four portions by gel filtration using a column (95 × 1.2 cm) of Sephadex G-10, eluting with 0.1 M HOAc. TLC indicates that product **3c** was separated from TEA–HOAc (R_f (G) 0.37; Nh). The fractions devoid of TEA were pooled and twice lyophilized. The residue was dissolved in a slight excess of 0.01 M HCl (4 × 50 mL) and lyophilized again. **3c**: total yield 1.03 g (1.47 mmol; 72% based on **3a**); mp (m) °C dec; TLC shows a major component (R_f (F) 0.03, R_f (G) 0.16; H_2SO_4, $KMnO_4$, Nh) and a minor component (R_f (F) 0.05, R_f (G) 0.22); H_2SO_4, $KMnO_4$, Nh); ^1H NMR (300 MHz), (Me_2SO-d_6) δ 8.06 (t, Gly NH_3^+, 2.6 (3) H, $^3J \sim 6$ Hz), 7.69 (s, amide NH, 0.7 (1) H), 4.13 (d, H-1, 3.1 (3) H, $J_{1,2}$ = 6.7 Hz, β-configuration), 4.09 (d, C (quat) CH_2, 3.1 (3) H, ^2J = 10.3 Hz), 3.70 (d, C (quat) CH_2, 3.1 (3) H, ^2J = 10.2 Hz), 3.62 (br s, H-4, 3.0 (3) H), 3.52[a] (m), and 3.44[b] (m, H-6A and H-6B, $J_{6A,6B}$ = 11.0 Hz, $J_{5,6A}$ = 6.0 Hz, $J_{5,6B}$ = 6.2 Hz) and ca. 3.5[c] (Gly CH_2), a + b + c (8.7 (8) H, 3.32[d] (m, H-5) and 3,27[e] (m, H-2 and H-3), d + e (8.7 (9) H). Signals of the OH groups were not observed, presumably due to the presence of (crystal bound) water in the sample. The singlet observed at 7.75 ppm (amide NH) can be assigned to the bisgalactoside analogue present as the minor component. ^{13}C NMR (Me_2SO-d_6) δ 176.10 (Gly C=O), 109.65 (C-1, β-configuration), 78.01 (C-5), 76.05 (C-3), 73.21 (C-2), 70.41 (C-4), 69.26 (CH_2O–Gal), 62.00 (C-6 and C (quat)), 38.71 (Gly CH_2). Anal, ($C_{40}H_{59}O_{27}N_2 \cdot 8H_2O$) C, H, N, H_2O.

N-Hydroxysuccinimidyl 5-Cholesten-3β-yloxy Succinate (4). To a solution of cholesteryl hydrogen succinate (5.0 g, 10.2 mmol) and *N*-hydroxysuccinimide (1.23 g, 10.7 mmol) in THF (40 mL), cooled to −10 °C, was added DCCI (2.40 g, 11.0 mmol). The mixture was stirred for 60 min at −10 °C and for another 180 min at room temperature and then left for 12 h at 4 °C. The precipitated *N,N'*-dicyclohexylurea was removed by filtration, the filtrate evaporated to dryness, and the residue crystallized from THF/2-propanol: yield 5.3 g (9.1 mmol, 89%); mp 142–144 °C; $[\alpha]_D$ −22.6° (*c* 1.4, THF); homogeneous on TLC, R_f (A) 0.52 (UV, H_2SO_4); ^1H NMR (100 MHz, $CDCl_3$) δ 5.33 (d, chol H-6, 1.0 (1) H), 4.55 (m, chol H-3, 1.0 (1) H), 2.76 (m, CH_2CH_2 of succinate and ONSu, 8.0 (8) H), 2.30–0.67 (other cholesteryl protons). The corresponding ^3H-labeled product was obtained by using 300 mg (0.616 mmol) ^3H-labeled cholesteryl hydrogen succinate (prepared as described above) and 110 mg (0.96 mmol) *N*-hydroxysuccinimide in 3 mL of THF: yield 227 mg (0.382 mmol, 62%).

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N-[Tris[(β-D-galactopyranosyloxy)methyl]methyl]-N$^{\alpha}$-[4-(5-cholesten-3β-yloxy)succinyl]glycinamide (5). Product **3c** (430 mg, 0.614 mmol) and *N*-ethylmorpholine (80 μL, 0.63 mmol) were dissolved in DMF (15 mL) with stirring. Subsequently, a solution of **4** (0.95 mmol) in DMA (19 mL) containing *N*-ethylmorpholine (83 μL, 0.66 mmol) was added. The mixture was stirred for 210 min at room temperature. Complete coupling was achieved as judged from TLC (50 μL of the mixture evaporated to dryness and dissolved in THF/water (2/1) for application on the thin-layer plate). The solution was acidified with HOAc (77 μL, 1.34 mmol) and evaporated to practically complete dryness. The product was precipitated by addition of THF (50 mL). The precipitate was collected on a glass filter, washed with THF (40 mL) and diethyl ether (40 mL), and dried above solid KOH in vacuo. A solution of the material in water was lyophilized. Yield: 603 mg of crude product. The above procedure was carried out once more with 0.648 mmol of **3c** as the starting material. TLC indicates the presence of a major product **5** and a minor product **6**, R_f (E) 0.21 and 0.40, R_f (F) 0.15 and 0.32, R_f (G) 0.55 and 0.63 (H_2SO_4, $KMnO_4$), together with two more polar components. Separation of the products **5** and **6** was achieved by chromatography of the mixture (930 mg in four portions) on a column of silica (9 × 6 cm; 115 g of silica particles of 40–63 μm) eluting with $CHCl_3$/methanol/water (54/40/6). Fractions containing either one of these two products were pooled and evaporated to dryness. Solutions of **5** or **6** in water were filtered (glass-fiber filter, Whatman GF/A) and twice lyophilized. Major product **5**: yield 534 mg (0.471 mmol, 50%, based on **3c**); $[\alpha]_D$ −9.7° (*c* 1, $CHCl_3/Me_2SO$, 1/1); homogeneous on TLC (R_f (E) 0.22, R_f (F) 0.12, H_2SO_4, $KMnO_4$); after hydrolysis of 32 μg (28.1 nmol) of **5** in 2 N HCl, 85 nmol of galactose (anthron assay for carbohydrate; 100.8% of expected value) and 25.2 nmol of cholesterol (89.7% of expected value) were found. **5**: ^{1}H NMR (300 MHz, $CDCl_3/Me_2SO$-d_6, 1/1) δ 7.99 (t, Gly NH, 0.9 (1) H, ^3J = 5.4 Hz), 7.07 (s, C (quat) NH, 1.0 (1) H), 5.34 (d, Chol H-6, 1.0 (1) H, ^3J = 4.2 Hz), 4.83 (d, Gal OH, 2.9 (3) H, ^3J = 4.6 Hz), 4.56[a] (d, Gal OH, ^3J = 5.2 Hz) and 4.53[b] (t, Gal OH-6, ^3J = 5.6 Hz) and 4.49[c] (m, Chol H-3), a + b + c (6.8 (7) H), 4.32 (d, Gal OH, 2.9 (3) H, ^3J = 4.6 Hz), 4.18 (d, Gal H-1, 3.2 (3) H, $J_{1,2}$ = 7.2 Hz, β-configuration), 4.14 (d, C (quat) CH_2, 2.9 (3) H, ^2J = 10.4 Hz), 3.86 (d, C (quat) CH_2, 2.9 (3) H, ^2J = 10.4 Hz), 3.73 (m, Gal H-4 and Gly CH_2, 5.1 (5) H), 3.61 (t, Gal H-6, 6.6 (6) H, ^3J = 5.8 Hz), 3.39 (m, Gal H-2, H-3 and H-5, 8 (9) H), 2.50 (m, succinate CH_2, 4.1 (4) H), 2.3–0.68 (other cholesterol protons, 41 (43) H); on addition of D_2O, the signals at 7.99, 7.07, 4.83, 4.56, 4.53, and 4.32 ppm disappear; ^{13}C NMR ($CDCl_3/Me_2SO$-d_6, 1/1) δ 171.67 (amide and ester C=O), 169.21 (amide C=O), 139.36 (Chol C-5), 122.16 (Chol C-6), 104.23 (Gal C-1, β-configuration), 75.14 (Gal C-5), 73.44 (Gal C-3 and Chol C-3), 70.95 (Gal C-2), 68.58 (Gal C-4), 67.99 (CH_2O–Gal), 61.00 (Gal C-6), 59.61 (C (quat)), 56.30 (Chol C-14), 55.70 (Chol C-17), 49.60 (Chol C-9), 30.03 and 29.45 (succinate CH_2), 42–11.68 (other cholesterol C atoms). Anal. ($C_{55}H_{93}O_{22}N_2 \cdot 8H_2O$) C, H, N, H_2O. Minor product **6**: yield 94 mg (0.097 mmol; 10% based on **3c**; homogeneous on TLC, R_f (E) 0.48, R_f (F) 0.28 (H_2SO_4, $KMnO_4$); ^{1}H NMR (300 MHz, $CDCl_3/Me_2SO$-d_6, 1/1) δ 8.05 (t, Gly NH, 1 (1) H), 7.18 (s, C (quat) NH, 0.9 (1) H), 5.34 (d, Chol H-6, 1.0 (1) H, ^3J = 4.2 Hz), 4.92 (d, Gal OH-a, 0.8 (1) H, ^3J = 3.9 Hz), 4.85 (d, Gal OH-a′, 0.9 (1) H, ^3J = 3.9 Hz), 4.73 (t, C (quat) CH_2OH, 0.9 (1) H, ^3J = 6.5 Hz), 4.60[a] (d) and 4.59[a] (d) (Gal OH-b and OH-b′, ^3J = 5.2 Hz), 4.56[b] (t) and 4.54[b] (t) (Gal OH-6 and OH-6′, ^3J = 5.2 Hz), 4.49[c] (m, Chol H-3), a + b + c (4.5 (5) H); 4.34 (br d, Gal OH-c and OH-c′, 1.8 (2) H, ^3J = 4.0 Hz), 4.18 (d) and 4.15 (d) (Gal H-1 and H-1′, 2.2 (2) H, $J_{1,2}$ = 7.4 Hz, β-configuration), 4.06 (d, CH_2O–Gal H_A, 1.0 (1) H, ^2J = 9.8 Hz), 3.99 (d, CH_2O–Gal H_A′, 1.0 (1) H, ^2J = 10.2 Hz), 3.75 (m, CH_2O–Gal H_B + H_B′, and Gly CH_2 and Gal H-4 + H-4′ and C (quat) CH_2OH, 8.3 (8) H), 3.61 (t, Gal H-6 + H-6′, 4.6 (4) H, ^3J = 5.9 Hz), 3.39 (m, Gal H-2 + H-2′ and H-3 + H-3′ and H-5 + H-5′, 5.3 (6) H), 2.50 (m, succinate CH_2, 3.8 (4) H), 2.3–0.68 (remaining Chol protons, 47 (43) H); on addition of D_2O, the signals at 8.05, 7.18, 4.92, 4.85, 4.73, 4.60, 4.59, 4.56, 4.54, and 4.34 ppm disappear; ^{13}C NMR ($CDCl_3/Me_2SO$-d_6) δ 171.64 (amide and ester C=O), 169.60 (amide C=O), 139.33 (Chol C-5), 122.13 (Chol C-6), 104.35 (Gal C-1, β-configuration), 75.20 (Gal C-5), 73.44 (Gal C-3 and Chol C-3), 70.89 (Gal C-2), 68.47 (Gal C-4), 68.09 and 67.98 (CH_2O–Gal), 61.37 (C (quat)), 60.85 (Gal C-6), 60,64 (C (quat) CH_2OH), 56.30 (Chol C-14), 55.66 (Chol C-17), 49.57 (Chol C-9), 42.75 (Gly CH_2), 29.93 and 29.39 (succinate CH_2), 42–11.68 (remaining Chol C atoms).

Compound **5** was also obtained with ^{3}H label in the cholesteryl moiety by reacting 182 mg (0.306 mmol) of ^{3}H-labeled **4** in 5.6 mL of DMF with 384 mg (0.545 mmol) of **3c** in 5.9 mL of DMF and 0.1 mL of *N*-ethylmorpholine. The desired compound was obtained by preparative thin-layer chromatography, using mixture E as mobile phase. The final product was homogeneous and had a specific radioactivity of 80 dpm/nmol.

Preparation of Liposomes and Leakage Determination. Small unilamellar liposomes containing sphingomyelin/cholesterol (1/1) having [^{3}H]inuline or [^{14}C]sucrose as marker for the internal water space were prepared as described before[11] with use of a Branson Sonifier B-12 at 60-W output. Leakage of label from the liposomes was determined by measuring the fraction of label that does not float to a density < 1.20 upon density gradient ultracentrifugation as performed in the manner described below. (As shown in Figure 2, all liposome cholesterol floats to density < 1.20).

Density Gradient Ultracentrifugation. Compound **5** (tritium labeled) was dissolved in a small volume of distilled water and added to 2 mL of a dispersion of liposomes (see above) or to 2 mL of citrated human plasma to a final concentration of up to 1 mM and in a proportion of up to 20 mol % of the total lipid content (including the compound itself). The mixture was left for 10 min at room temperature in a polyallomer centrifuge tube. Thereafter, 975 mg of solid KBr was added and 1 mL of water was used to rinse all the KBr crystals to the bottom of the tube. After complete dissolution of all KBr, the tube was filled by the consecutive layering of 3-mL portions of 9%, 3%, and 1.1% (w/v) NaCl solutions. The tubes were placed in the 6 × 14 swing-out rotor fitting in the MSE superspeed ultracentrifuge. The rotor was operated for 20 h at 20 °C, at 39 000 rpm. After ending the run, fractions of 0.5 mL were taken with a manually operated suction pipet, starting from the meniscus. From these fractions, aliquots were taken for determinations of cholesterol content[26] of radioactivity and of the density (in a DMA apparatus, equipped with a microcuvette, Anton Paar),

Studies with Labeled Liposomes and LDL in the Rat in Vivo. LDL isolated from human plasma at density 1.019 < *d* < 1.063 was labeled with ^{125}I according to the procedure of Bilheimer et al.[27] An amount of LDL corresponding with 20 μg of LDL-protein was mixed with 0, 5, or 13 μg of substance **5** in water, incubated for 10 min at room temperature, and thereafter injected intravenously in male rats, anesthesized with pentobarbital. In independent experiments we found all **5** to be associated with the LDL when mixed and incubated in these conditions (Figure 4 in the supplementary material). Ten minutes after injection, blood was taken by aortic puncture and the liver was perfused through the portal vein with Ca^{2+}-free Hanks buffer at a flow rate of 14 mL/min. Eight minutes after the start of the perfusion, the liver was excised. Radioactivity in serum and liver samples was determined in a γ-counter. Results were used to calculate the amount of label in total blood (assuming 4 mL of serum per 100 g of body weight) and liver, which are expressed in percent of the injected dose. Where specified, 110 mg of 2-acetamido-2-deoxygalacto- or -glucopyranose was injected intravenously 1 min before administration of the labeled LDL. Experiments with liposomes, containing [^{14}C]sucrose untreated or preloaded with **5** (10 mol %), were carried out as described before.[11]

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Registry No. 1, 19285-38-2; 2, 91202-74-3; **3a**, 91202-75-4; **3b**, 91202-77-6; **3c**, 91202-76-5; **4**, 88848-79-7; 4 (tritium labeled), 91202-78-7; **5**, 91202-79-8; **5** (tritium labeled), 91202-80-1; **6**,

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91208-73-0; [1α,2α-$^3H_2$]-cholesteryl hydrogen succinate, 91202-72-1; [1α,2α-$^3H_2$]-cholesterol, 27246-11-3; succinic anhydride, 108-30-5; [1α,2α-$^3H_2$]-cholesteryl hydrogen succinate ammonium salt, 91202-73-2; penta-*O*-acetyl-β-D-galactopyranose, 4163-60-4; Tris, 77-86-1; *N*-(benzyloxycarbonyl)glycine, 1138-80-3; cholesteryl hydrogen succinate, 1510-21-0; *N*-hydroxysuccinimide, 6066-82-6.

Supplementary Material Available: Figure 4 showing the results of density gradient ultracentrifugation of **5** in the presence of LDL (2 pages). Ordering information is given on any current masthead page.

Molecular Analysis of Hexahydro-1*H*-indeno[1,2-*b*]pyridines: Potential Antidepressants

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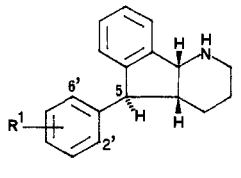
Biological tests indicate hexahydro-1*H*-indeno[1,2-*b*]pyridines to be potential human antidepressants with additional stimulating properties. Two diastereomeric series with H_{4a},H_5-trans,H_{4a},H_{9b}-cis and H_{4a},H_5-cis,H_{4a},H_{9b}-cis configurations have been tested biologically. The results revealed that the H_{4a},H_5-cis,H_{4a},H_{9b}-cis series and the ortho-substituted 5-phenyl H_{4a},H_5-trans,H_{4a},H_{9b}-cis compounds lack activity. Neither the conformation with lowest potential energy nor any other electron-derived parameter correlate with these data. The only relevant difference between the active and the inactive compounds detected thus far is the rotational barrier of the phenyl in the 5-position. The conclusion was reached that certain conformations, which do not resemble those of lowest potential energy, cannot be adopted by the inactive compounds. Therefore, the interaction of the drug with the binding site, responsible for its biological activity, appears to be governed by a dynamic process. This process is characterized by a transformation of the conformation of lowest potential energy to one with an energy content above the minimum.

The easy accessibility[1] of 2,3,4,4a,5,9b-hexahydro-1*H*-indeno[1,2-*b*]pyridines with the H_{4a},H_5-trans,H_{4a},H_{9b}-cis (**1**) and H_{4a},H_5-cis,H_{4a},H_{9b}-cis configuration (**2**) made it possible to perform intensive studies on structure–activity relationships of these two diastereomeric series.[2] Compounds related to **1** were found to be strong tetrabenazine antagonists with stimulating properties, activity being almost independent of the substituents introduced into the aromatic ring at the 5-position. The only exception was the lack of activity of compounds bearing a substituent in the ortho position of the phenyl ring (**1**, R^1 = 2-CH_3). In addition we found that compounds related to **2** lack activity, independent of R^1. Compound **2** (R^1 = 4-NH_2) is the only exception to this rule; however, it is assumed that the special character of the amino group in comparison to other substituents overrules any other common geometrical derived parameter that may explain the inactivity of this series. Because of the almost clear-cut structure–activity relationships, the current study was undertaken to find relevant molecular parameters that might correlate with the experimental biological data and that could explain the biological findings.



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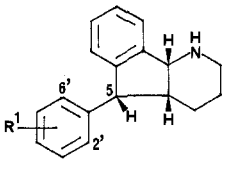

2

Method. Conformational analyses of the compounds selected was done with the computer program SCRIPT.[3] The main features of SCRIPT are as follows: generation of three-dimensional structures from the two-dimensional representation of a given compound, calculation of the energies of the generated conformers, energy minimization of these conformers, and a manipulation phase in which one can analyse precisely the geometry of the final structures.

The initial generation of the three-dimensional structures is based primarily on the division of the molecules into chain and ring fragments. These main parts are described by sets of torsion angles that allow the calculation of all conformational states for the chain and the ring fragments. The combinatory product of the conformational diagrams for rings and chains produces all possible conformational diagrams of the molecule. From these diagrams the three-dimensional coordinates are calculated with use of numerical values for atomic distances, valency angles, and torsion angles.

After constructing the three-dimensional conformers, it is possible to calculate the corresponding molecular energies. The energy is the sum of stretching, bending, torsional, van der Waals, electrostatic, and hydrogen-bonding energy terms. Each of the conformers generated initially is now relaxed with respect to all its degrees of internal freedom in order to reach the corresponding minimum on the conformational potential surface. By definition, minimum conformational energy is reached when the sum of stretching, bending, torsional, van der Waals, electrostatic, and hydrogen-bonding energies does not change during an iteration step more than the preselected convergence criteria. In a typical procedure 5000 iteration steps and a convergence criteria of 0.01 kcal/mol were selected. As an example, the total energy gain during a minimization procedure as the sum of the different terms is listed in Table I for compound **1** (R^1 = 4-CH_3) and compound **2** (R^1 = 4-CH_3). For further details of the program see ref 3.

Generation of the Conformers. For the calculations three compounds were selected from each of the diastereomeric series bearing methyl groups in the para, meta, or ortho position. With a chair conformation for the piperidine ring, the program generates two conformations for the condensed three system (Figure 1, conformations 1 and 2).

The differences between these two possible conformations can easily be seen. C-5 and C-9b take in each case a position in the same plane as the benzo ring, whereas

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(3) N. C. Cohen, P. Colin, and G. Lemoine, *Tetrahedron*, 37, 1711 (1981).

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