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Lysine-based Cluster Mannosides That Inhibit Ligand Binding to the Human Mannose Receptor at Nanomolar Concentration*

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In search of synthetic high affinity ligands for the mannose receptor, we synthesized a series of lysine-based oligomannosides containing two (M_2L) to six (M_6L_5) terminal α-D-mannose groups that are connected with the backbone by flexible elongated spacers (16 Å). The synthesized cluster mannosides were all able to displace binding of biotinylated ribonuclease B and tissue-type plasminogen activator to isolated human mannose receptor. The affinity of these cluster mannosides for the mannose receptor was continuously enhanced from 18–23 μM to 0.5–2.6 nM, with mannose valencies increasing from two to six. On average, expansion of the cluster mannoside with an additional α-D-mannose group resulted in a 10-fold increase in its affinity for the mannose receptor. M_3L_2 to M_6L_5 displayed negative cooperative inhibition of ligand binding to the mannose receptor, suggesting that binding of these mannosides involves multiple binding sites. The nanomolar affinity of the most potent ligand, the hexamannoside M_6L_5 makes it the most potent synthetic cluster mannoside for the mannose receptor yet developed. As a result of its high affinity and accessible synthesis, M_6L_5 not only is a powerful tool to study the mechanism of ligand binding by the mannose receptor, but it is also a promising targeting device to accomplish cell-specific delivery of genes and drugs to liver endothelial cells or macrophages in bone marrow, lungs, spleen, and atherosclerotic plaques.

The mannose receptor is a 175-kDa membrane-associated protein that is localized on sinusoidal liver cells, peripheral and bone marrow macrophages, and dendritic cells (1–4). It recognizes and internalizes mannosylated polysaccharides from pathological microorganisms (5), tumor cells (6), and yeast cells (7) and glycoproteins like type-I procollagen (8), tissue-type plasminogen activator (9), or various lysosomal enzymes (10). As such, the mannose receptor participates in the nonimmune host-defense system. In addition, the macrophage receptor is implicated in major histocompatability complex-mediated antigen presentation by dendritic cells (11).

The cDNA of the mannose receptor has been sequenced by Taylor *et al.* (12) and codes for five types of domains (13): an N-terminal cysteine-rich domain, a transmembrane domain, a fibronectin type II-like domain, a domain composed of eight strongly homologous repeats (the so-called carbohydrate recognition domains or CRDs)[1] and a C-terminal cytoplasmic tail. Taylor and Drickamer (13, 14) have established that the CRDs are involved in ligand binding. Recent structure-function studies of recombinant truncated forms of the mannose receptor provided new insight into the mechanism of ligand binding by the mannose receptor (13, 14). On basis of these results, it was proposed that CRD4 is the only CRD to display a monosaccharide specificity characteristic for the mannose receptor (15). CRD4 and CRD5 appear to be required for high affinity binding of high mannose-type glycoproteins and mannosylated bovine serum albumin (BSA). By contrast, at least five consecutive CRDs are needed for avid binding of highly mannosylated polysaccharides like mannan (14). Since each CRD embeds only a single mannose binding site, this implies that the mannose receptor may accommodate five to eight appropriately configured terminal α-D-mannose residues. Previous studies by Hoppe *et al.* (16) and Jansen *et al.* (17) established that the affinity of mannosylated albumin for the mannose receptor indeed correlated with the extent of mannosylation. Surprisingly, derivatization with more than 22–24 mannose groups was required for high affinity recognition by the mannose receptor. This is considerably higher than the maximum number of CRDs thought to participate in ligand binding. On one hand the above finding may reflect a purely entropic phenomenon, caused by an increased chance of α-D-mannose groups to be adequately configured with respect to each other. On the other hand, it may suggest that multiple receptor molecules cooperate in the ligand binding process.

To address this we have evaluated the effect of mannose valency on the affinity for the mannose receptor in close detail using a series of homologous cluster mannosides that possess two to six terminal α-D-mannose groups. This study shows that recognition of low molecular weight mannosides by the mannose receptor is consistently, and not in a stepwise manner, enhanced with valencies increasing from two to six and thus provides new information on the process of ligand binding by the mannose receptor.

EXPERIMENTAL PROCEDURES

Materials

$Na^{125}I$ in 0.1 M NaOH (13.5 mCi/μg) was purchased from Amersham (Buckinghamshire, United Kingdom). BSA (fraction V, delipidated), collagenase (type I), *p*-nitrophenolphosphate, *p*-aminophenyl-α-D-mannopyranoside, and ribonuclease B (bovine pancreas) were purchased

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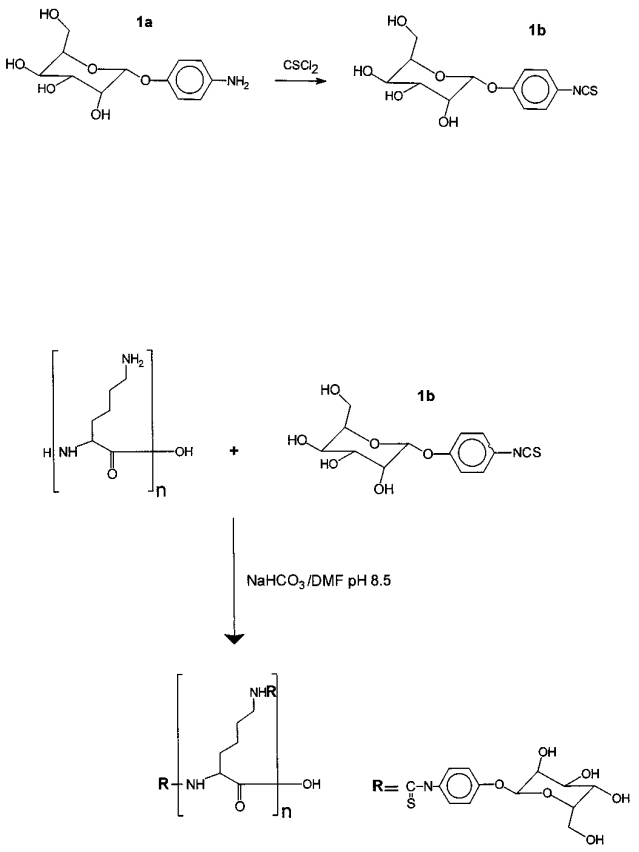
[1] The abbreviations used are: CRD, carbohydrate recognition domain; t-PA, tissue-type plasminogen activator; bio-t-PA, biotinylated t-PA; Ribo B, ribonuclease B; bio-Ribo B, biotinylated Ribo B; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium.



SCHEME 1. **Reaction scheme of the synthesis of cluster mannosides 2a to 2e. 2a**, $n = 1$; **2b**, $n = 2$; **2c**, $n = 3$; **2d**, $n = 4$; **2e**, $n = 5$.

from Sigma. L-Lysine-HCl; L-lysyl-L-lysine, 2HCl, 0.5 H_2O; di-(L-lysyl)-L-lysine, 3AcOH; tri-(L-lysyl)-L-lysine, 4AcOH; and tetra-(L-lysyl)-L-lysine, 5AcOH were all obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Thiophosgene was obtained from Aldrich Chemie (Bornem, Belgium). *N,N*-Dimethylformamide obtained from Merck (Hohenbrunn, Germany) was refluxed for 4 h with CaH_2 (5 g/liter) and distilled under reduced pressure. Streptavidin-alkaline-phosphatase conjugate was from Amersham. Tissue-type plasminogen activator (t-PA) from a recombinant human melanoma cell culture was provided by Dr. J. Verheijen (Gaubius Laboratory, TNO-PG, Leiden, The Netherlands).

Chromatography

Thin layer chromatography was performed using silica F_{254} preformed layers (0.1 mm) on a plastic backing (Schleicher & Schuell DC-F1500) and MeOH (A), acetonitrile-H_2O (80:20, v/v) (B), or MeOH-$CHCl_3$ (80:20, v/v) (C) as eluent. Carbohydrates were visualized after spraying with 20% H_2SO_4 in MeOH and subsequent heating at 140–160 °C. Amine- or amide-containing compounds were visualized after spraying with ninhydrin and subsequent heating at 140 °C, while compounds containing an aromatic group were visualized by UV shadowing (254 nm). Preparative column chromatography was performed using Kieselgel 60 (Merck, Hohenbrunn, Germany).

Instrumental Analysis

NMR spectra were recorded at 300 MHz (^{1}H) with a Bruker WM300 spectrometer operating in the Fourier transform mode. Chemical shifts are denoted in ppm (δ) relative to tetramethylsilane as internal standard. Mass spectra were obtained using a Finnigan MAT 900 mass spectrometer, using electrospray (MeOH/H_2O (80/20) + 1% HAc) as the ionization technique. Positive ions were visualized after data processing using the HMR BSCAN UP profile.

Synthesis

*4-(α-D-Mannopyranosyloxy)phenylisothiocyanate (**1b**)*—Compound **1a** (Scheme 1) was converted into the phenyl isothiocyanate derivative (**1b**) as described by Monsigny *et al.* (18) and Kataoka *et al.* (19). In short, a mixture of compound **1a** (244 mg, 0.9 mmol) and thiophosgene (0.52 ml, 5.1 mmol) in ethanol/H_2O (80:20, v/v; 50 ml) was stirred for 2 h at room temperature. The excess of thiophosgene was removed by perspiration with N_2 for 1 h at room temperature. After concentration of the solution under reduced pressure, the residue was dissolved in a small volume of distilled water (1–2 ml), and NaOH (1.0 M) was added to pH 6.0. The solution was concentrated, and the residue was chromatographed over a Kieselgel 60 column (40 ml) using CH_2Cl_2/MeOH (80:20, v/v) as eluent. Fractions containing product **1b** were pooled and lyophilized to yield 288 mg of a white crystalline powder (0.916 mmol; 102%); R_F, 0.75 (B), 0.67 (C). Mass: 313.1 (M^+ calculated: 313.32); ^{13}C NMR Attached Proton Test (APT): 161.3 (C_1-phenyl), 138.5 (C_4-phenyl), 127.8 (C_2, 6-phenyl), 118.9 (C_3, 5-phenyl), 101.1 (C_1-Man, α-configuration), 76.9 (C_5-Man), 73.6 (C_2-Man), 72.1 (C_3-Man), 70.0 (C_4-Man), 63.3 (C_6-Man).

Mannosylated Oligolysines—Mannosylated (oligo)lysines, *i.e.* M_2L (**2a**), M_3L_2 (**2b**), M_4L_3 (**2c**), M_5L_4 (**2d**), and M_6L_5 (**2e**) were synthesized according to the procedure of Jansen *et al.* (17) with slight modifications. In general, compound **1b** (0.10 mmol; 32 mg) and (oligo)lysine (0.83 molar equivalents on the basis of the amino group content) were dissolved in a mixture of 0.1 M sodium hydrogen carbonate (pH 8.5) and *N,N*-dimethylformamide (50:50, v/v; 4 ml), and the solution was stirred for 18 h in the dark at room temperature. The progression of the reaction was monitored by TLC. In case all of compound **1b** had reacted, while the reaction was not yet complete, an additional amount of **1b** (0.4 molar equivalents) was added, and the reaction mixture was incubated for another 8 h. Subsequently, the mixture was concentrated *in vacuo*, and the crude products **2a**–**2e** were chromatographed over a Kieselgel 60 column using acetonitrile/H_2O (80:20, v/v) as eluent. Fractions containing products **2a**–**2e** were pooled and lyophilized to yield a whitish powder.

N^2,N^6-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysine (M_2L; 2a)—R_F = 0.31 (A); yield: 5.2 μmol (12.5%); mass (M + Na^+): 794.6 (M^+ calculated: 771.5); ^{1}H NMR (D_2O/CD_3OD): δ 1.27 (q, br, 2H, CH_2-γ), 1.63 (q, br, 2H, CH_2-δ), 1.90 (q, br, 2H, CH_2-β), 3.50 (t, br, 2H, CH_2-ε), 3.75–4.13 (m, 11H, Man H-1 to H-5 and CH-α); 4.04 (d, br, Man H-1(α), J = 3.2 ppm); 5.61 (dd, 4H, Man H-6 and H-6′), 7.17 (m, 8H, phenyl-CH).

*N^2-[N^2,N^6-Bis [N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N- (p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysine (M_3L_2; **2b**)*—R_F = 0.20 (A); yield: 11.1 μmol (40%); mass (M + Na^+): 1235.8 (M^+ calculated: 1212.8); ^{1}H NMR (D_2O/CD_3OD): δ 1.33 (q, 4H, CH_2-γ), 1.54 (q, br, 4H, CH_2-δ), 1.76 (q, br, 4H, CH_2-β), 3.39 (t, br, 4H, CH_2-ε), 3.73–4.19 (m, 17H, Man H-1 to H-5 and CH-α); 4.06 (d, br, Man H-1(α), J = 3.1 ppm); 5.57 (dd, 6H, Man H-6 and H-6′), 7.22 (m, 12H, phenyl-CH).

*N^2-[N^2-[N^2,N^6-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysine (M_4L_3; **2c**)*—R_F = 0.11 (A); yield: 7.0 μmol (34%); mass (M + Na^+): 1676.1 (M^+ calculated: 1654.1); ^{1}H NMR (D_2O/CD_3OD): δ 1.26 (q, 6H, CH_2-γ), 1.57 (q, br, 6H, CH_2-δ), 1.90 (q, 6H, CH_2-β), 3.31 (t, br, 6H, CH_2-ε), 3.68–4.26 (m, 23H, Man H-1 to H-5 and CH-α), 3.97 (d, br, Man H-1(α), J = 2.9 ppm); 5.52 (dd, 8H, Man H-6 and H-6′), 7.05 (m, 16H, phenyl-CH).

*N^2-[N^2-[N^2-[N^2,N^6-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)-anilino)thiocarbamyl]-L-lysine (M_5L_4; **2d**)*—R_F = 0.05 (A); yield: 11.5 μmol (50%); mass (½M^{2+} + Na^+): 1072.6 (M^+ calculated: 2095.4); ^{1}H NMR (D_2O/CD_3OD): δ 1.38 (q, br, 8H, CH_2-γ), 1.75 (q, br, 8H, CH_2-δ), 1.88 (q, 8H, CH_2-β), 3.34 (t, br, 8H, CH_2-ε), 3.66–4.36 (m, 29H, Man H-1 to H-5 and CH-α); 3.95 (d, br, Man H-1(α), J = 2.5 ppm); 5.47 (dd, 10H, Man H-6 and H-6′), 7.05 (m, 20H, phenyl-CH).

*N^2-[N^2-[N^2-[N^2-[N^2,N^6-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^6-[N-(p-(α-D-mannopyranosyloxy)-anilino)thiocarbamyl]-L-lysine (M_6L_5; **2e**)*—R_F = 0.05 (A); yield: 11.5 μmol (83%); mass (½M^{2+} + Na^+): 1291.3 (calculated: 2536.7); ^{1}H NMR (D_2O/CD_3OD): δ 1.32 (q, br, 8H, CH_2-γ), 1.52 (q, br, 8H, CH_2-δ), 1.74 (q, 8H, CH_2-β), 3.43 (t, br, 8H, CH_2-ε), 3.65–4.28 (m, 29H, Man H-2 to H-5 and CH-α), 3.91 (d, br, Man H-1(α), J = 2.7 ppm); 4.92 (dd, 10H, Man H-6 and H-6′), 7.06–7.10 (m, 20H, phenyl-CH).

Biotinylation or Radioiodination of t-PA and Ribonuclease B

Ribonuclease B or t-PA were dialyzed against 0.1 M $NaHCO_3$, pH 8.5, and subsequently incubated for 3 h at room temperature with *N*-hydroxysuccinimide-activated biotin (Zymed Laboratories Inc., South San Francisco, CA) at a ratio of 1 mol of protein to 6 mol of *N*-hydroxysuccinimide-activated biotin for ribonuclease B and 200 mol of *N*-hydroxysuccinimide-activated biotin for t-PA, respectively. After the reaction, the protein was dialyzed against 20 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl, 5 mM $CaCl_2$, and 0.5% (v/v) Tween 80. t-PA was radioiodinated by the IODO-GEN method as described, and a specific radioactivity of 3500–5000 cpm/ng of protein was obtained (20).

Isolation of Endothelial Liver Cells

Rat endothelial liver cells were isolated by a collagenase perfusion protocol at 37 °C as described previously (21). Liver cells were separated by differential centrifugation, and endothelial cells were subsequently purified by counterflow centrifugation. The purity of endothelial cells as monitored by peroxidase staining was at least 95%. Viability of the cells used for *in vitro* experiments was judged by 0.2% trypan blue exclusion.

Isolation of Human Mannose Receptor

Human mannose receptor was isolated from human placenta after solubilization with Triton X-100 and subsequent affinity chromatography over mannosylated albumin-Sepharose according to Otter *et al.* (22).

Mannose Receptor Binding Assay

Displacement studies of binding of biotinylated ribonuclease B and biotinylated t-PA to isolated human mannose receptor were performed essentially as described by Otter *et al.* (9). Activated PVC multiwell plates (Flow Laboratories) were coated overnight at 4 °C with purified mannose receptor (±15 ng/well) in coating buffer (100 μl; pH 7.4), containing 20 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$. After washing, the wells were incubated for 30 min at 20 °C with assay buffer (coating buffer supplemented with 0.5% Tween 80 and 0.1% BSA) (125 μl) to minimize aspecific ligand binding. Then the wells were washed and preincubated for 30 min at 20 °C with a solution of α-D-mannose (10 μM to 100 mM) or one of the cluster mannosides (1 nM to 2 mM) in assay buffer (100 μl). Biotinylated ribonuclease B or biotinylated t-PA in assay buffer was added to a final concentration of 580 and 1.3 nM, respectively, and the mixture was incubated for 2 h at 20 °C. After a washing step, the wells were incubated for 1 h at room temperature with streptavidin-alkaline phosphatase conjugate (1:1000 dilution in assay buffer; 100 μl). *p*-Nitrophenolphosphate (1 mg/ml; 100 μl in a 100 mM diethanolamine, 5 mM $MgCl_2$ buffer, pH 9.5) was added after thorough rinsing of the wells. The plate was incubated for 4 h at 25 °C, during which the ΔA_{405}/h was determined as a measure of ligand binding. Uncoated wells were used as a control for aspecific adherence of biotinylated ribonuclease B or biotinylated t-PA to the wells.

Competition Studies of ^{125}I-t-PA Binding to Endothelial Liver Cells

Competition studies of ^{125}I-t-PA binding to endothelial liver cells were performed as described before (23). Endothelial cells (2×10^6; 150 μg of cell protein), in Dulbecco's modified Eagle's medium (DMEM, 0.5 ml), containing 2% (w/v) BSA and ^{125}I-t-PA (1 nM), were incubated for 2 h at 4 °C with a variable concentration of unlabeled biotinylated t-PA (bio-t-PA), biotinylated Ribo B (bio-Ribo B), or M_6L_5 ranging from 0.1 nM to 20 μM. Following incubation, cells were washed twice with DMEM, 0.2% BSA and once with DMEM, and the cell-associated radioactivity was counted. Nonspecific binding was defined as ^{125}I-t-PA binding in the presence of 100 mM α-D-mannose. Cell-bound radioactivity was determined using a Packard γ-counter and corrected for protein content.

Data Analysis

To calculate the IC_{50} values and Hill coefficients, the displacement binding data were analyzed according to the following binding model: percentage of specific binding = $100/(1 + ([\text{displacer}]/IC_{50}{}^{nH})$ using a computerized nonlinear fitting program (Prism, ISI software; Ref. 24). As a measure of the actual affinity for the mannose receptor, apparent inhibition constants (dissociation constants) were calculated from the IC_{50} values using the equation $K_{i\,(app)} = IC_{50}/(1 + [\text{ligand}]/K_d)$ and using K_d values for biotinylated ribonuclease B and t-PA obtained from the saturation binding studies (550 and 1.66 nM, respectively). The irregular competition curves of α-D-mannose were also fitted according to a two-site mixed stimulation/inhibition model (percentage of specific binding = (100 + percentage of maximal stimulation × (1 − 1/(1 + [displacer]/SC_{50})))/(1 + [displacer]/IC_{50}), SC_{50} being the concentration at which half-maximal stimulation of ligand binding to the mannose receptor is attained. The significance of the differences between means was tested by unpaired two-way Student's *t* test.

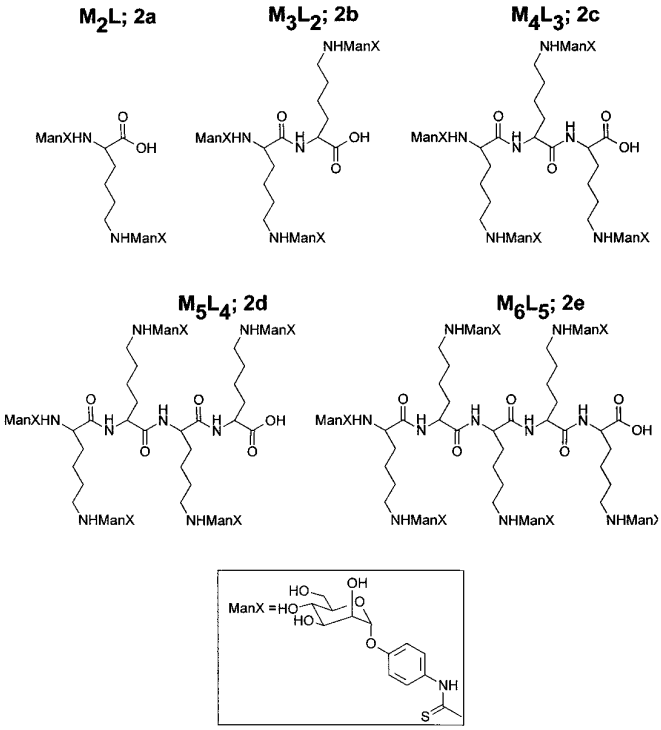


FIG. 1. **Chemical structures of the cluster mannosides 2a to 2e.**

RESULTS

Synthesis of the Cluster Mannosides—The cluster mannosides were prepared by a two-step synthetic procedure. First, 4-aminophenyl-α-D-mannopyranoside (**1a**) was quantitatively converted into 4-(α-D-mannopyranosyloxy)phenylisothiocyanate using thiophosgene (**1b**). Second, the activated α-D-mannopyranoside **1b** was reacted with a series of oligolysines, *i.e.* mono-, di-, tri-, tetra-, and pentalysine. Progression of the coupling was monitored on TLC by ninhydrin staining. The reaction was considered to be complete when no significant ninhydrin-positive spots, indicative of unreacted amino groups, could be detected. Subsequent chromatography of the crude reaction mixture over Kieselgel 60 and lyophilization afforded products **2a**–**2e** as white crystalline powders at yields ranging from 34 to 83%. TLC analysis (UV, ninhydrin, sulfuric acid detection) confirmed that the isolated products **2a**–**2e** were fully mannosylated, did not contain any free amino groups, and were apparently pure. ^{1}H NMR and mass spectrometry of the isolated products **2a**–**2e** were in agreement with the chemical structure of the anticipated products M_2L, M_3L_2, M_4L_3, M_5L_4, and M_6L_5 (for chemical structures, see Fig. 1).

Competition Binding Studies—First we have performed saturation studies of bio-Ribo B and bio-t-PA binding to the isolated mannose receptor in the enzyme-linked receptor sorbent assay described by Otter *et al.* (9) and Barret-Bergshoeff *et al.* (25) (Fig. 2). Bio-Ribo B and bio-t-PA binding appears to be saturable (0.354 ± 0.006 Δ*A*/h and 0.305 ± 0.003 Δ*A*/h, respectively) and obeyed classical law of mass action kinetics. The maximal binding capacity of bio-Ribo B and bio-t-PA were comparable. Analysis of the binding curves gave dissociation constants of 550 ± 70 nM for bio-Ribo B and 1.66 ± 0.05 nM for bio-t-PA. Hill coefficients were calculated to be close to unity

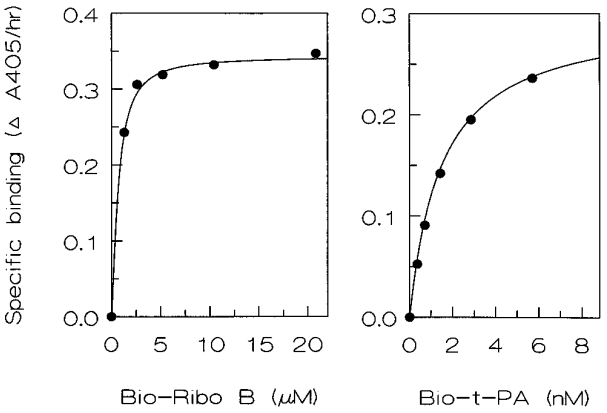


FIG. 2. **Saturation curve of bio-Ribo-B binding (*left panel*) and bio-t-PA (*right panel*) binding to isolated human mannose receptor in a receptor sorbent assay.** Multiwells, coated with isolated human mannose receptor, were incubated for 2 h at 20 °C with a variable concentration of biotinylated ribonuclease B or biotinylated t-PA. After incubation, ligand binding was determined as described under "Experimental Procedures." Specific binding of bio-Ribo B and bio-t-PA is plotted as ΔA/h and is corrected for aspecific binding determined in the presence of uncoated wells. The saturation binding curves were fitted using computerized nonlinear regression procedures.

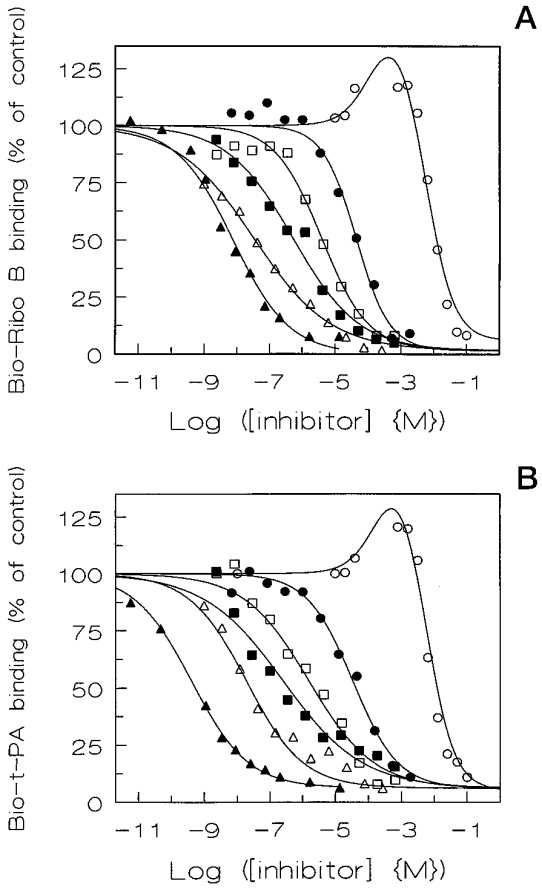


FIG. 3. **Competition studies of binding of biotinylated ribonuclease B (*A*) or biotinylated t-PA (*B*) to the isolated human mannose receptor by the following mannosides: α-D-mannose (○); M_2L (●); M_3L_2 (□); M_4L_3 (■); M_5L_4 (△), and M_6L_5 (▲).** Multiwells, coated with isolated human mannose receptor, were incubated for 2 h at 20 °C with a fixed concentration of biotinylated ribonuclease B (580 nM) or biotinylated t-PA (1.3 nM) in the absence or presence of displacer, at concentrations ranging from 10^{-10} to 10^{-1} M. After incubation, ligand binding was determined as described under "Experimental Procedures." Total binding of bio-Ribo B and bio-t-PA is plotted as percentage of the control (without displacer) *versus* the log of the displacer concentration (in M). The inhibition curves for α-D-mannose, M_2L, M_3L_2, M_4L_3, M_5L_4, and M_6L_5 were fitted using computerized nonlinear regression procedures.

(1.16 ± 0.43 and 1.13 ± 0.03, respectively), indicative of ligand binding to a single binding site. Subsequently, competition studies of bio-Ribo B binding were performed for the synthesized mannosides. As can be seen from Fig. 3*A*, all of the tested mannosides were able to completely inhibit the specific binding of bio-Ribo B to the isolated human mannose receptor. The potency of the compounds to inhibit bio-Ribo B binding was significantly increased with increasing mannose valency. The apparent inhibition constant $K_{i\,(app)}$ of α-D-mannose was at least 10^6-fold higher than that of the hexamannoside M_6L_5, the most potent ligand in this study (2.7 mM and 2.6 nM, respectively, Table I). Even the dimannoside M_2L possessed a considerably higher affinity ($K_{i\,(app)}$ = 17.5 μM) than α-D-mannose.

To verify that the cluster mannosides inhibited bio-Ribo B binding in a competitive fashion by blocking the mannose binding site on the mannose receptor rather than by directly interfering with bio-Ribo B itself, competition studies were also performed using another ligand for the human mannose receptor, bio-t-PA (22) (Fig. 3*B*). In data not shown here, it was demonstrated that bio-t-PA binding to the mannose receptor was fully inhibited in a competitive fashion by ribonuclease B, indicating that both ligands interact with the same binding site on the mannose receptor. The bio-t-PA competition curves clearly demonstrated that the potency of the cluster mannosides to inhibit bio-t-PA binding was analogously increased with increasing mannose valency. $K_{i\,(app)}$ values calculated from the bio-t-PA competition curves ranged from 2.8 mM for α-D-mannose to 8.9 nM for M_5L_4 and 0.5 nM for M_6L_5. This is illustrated in Fig. 4, in which $-\log(IC_{50})$ values of the mannosides from the bio-Ribo B assay are plotted against those from the bio-t-PA assay. Clearly, the affinities in both assays correlate excellently (correlation coefficient 0.984; $p < 0.0001$; slope 1.11 ± 0.07). Fig. 5 shows that there is only a minor tendency of the $-\log(IC_{50})$ values to level off at high mannose valencies, suggesting that the cluster mannosides did not yet reach the theoretical maximum affinity. Moreover, the increase in affinity is steady and intermediate plateau values for the cluster mannosides were not observed.

To calibrate the receptor sorbent assay, competition studies of ^{125}I-t-PA binding to endothelial liver cells were performed for M_6L_5, bio-t-PA, and bio-Ribo B. All three tested ligands were able to inhibit ^{125}I-t-PA binding (Fig. 6) at inhibition constants that were congruent to those found in the receptor sorbent assay ($K_{i\,(app)}$ = 1.7 ± 0.2, 3.4 ± 0.4, and 190 ± 70 nM, respectively). This further substantiates the validity of $K_{i\,(app)}$ values derived from this receptor sorbent assay.

Interestingly, displacement of bio-Ribo B and bio-t-PA binding to the human mannose receptor by the mannosides was not monophasic, as judged from the low Hill coefficients of M_3L_2, M_4L_3, M_5L_4, and M_6L_5 (n_H = 0.4–0.5) (Fig. 7). In contrast, saturation curves of bio-Ribo B and bio-t-PA binding to isolated mannose receptor proceeded in a purely competitive fashion (n_H = 1.16 ± 0.43 and 1.17 ± 0.02, respectively). In both assays, the bivalent mannoside M_2L exhibited an intermediate Hill coefficient of 0.63 ± 0.05 (bio-Ribo B) and 0.85 ± 0.1 (bio-t-PA). The Hill coefficients of both ligand binding assays correlated significantly (r^2 = 0.990, slope 1.006 ± 0.05; $p < 0.0002$). Competition curves of α-D-mannose were clearly irregular. α-D-Mannose appeared to stimulate ligand binding to the mannose receptor by ±25% at concentrations ranging from 30 to 300 μM. At higher concentrations, α-D-mannose inhibited binding in a positive cooperative fashion with Hill coefficients of 2.1 ± 0.5 (bio-Ribo B) and 2.2 ± 0.6 (bio-t-PA). Competition studies of 4-aminophenyl-α-D-mannopyranoside also gave a

TABLE I

Affinity of the cluster mannosides as determined from competition studies of binding of biotinylated ribonuclease B or t-PA to the isolated mannose receptor

$-\text{Log}(IC_{50})$ values (±S.D.) and $K_{i\ (app)}$ values were calculated from the displacement curves using nonlinear regression analysis.

Compound	Bio-Ribo B		Bio-t-PA	
	$-\text{Log}(IC_{50})$ (±S.D.)	$K_{i\ (app)}$	$-\text{Log}(IC_{50})$ (±S.D.)	$K_{i\ (app)}$
		nM		nM
α-D-Mannose	2.00 (±0.04)	2,700,000	1.98 (±0.04)	2,800,000
M_2L	4.49 (±0.07)	17,500	4.38 (±0.07)	22,600
M_3L_2	5.76 (±0.10)	950	5.43 (±0.09)	2020
M_4L_3	6.61 (±0.15)	135	6.30 (±0.09)	270
M_5L_4	7.56 (±0.08)	15.1	7.78 (±0.07)	8.9
M_6L_5	8.33 (±0.09)	2.6	9.05 (±0.09)	0.5

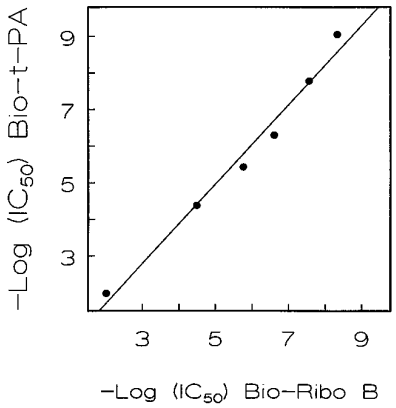


FIG. 4. **Correlation between the potency of the mannosides to inhibit bio-t-PA and ribonuclease B binding to the isolated mannose receptor.** The inhibitory potencies of the cluster mannosides are derived from the data in Table I and are expressed as $-\log(IC_{50}) \pm$ S.D. Invisible error bars indicate errors smaller than the *symbol* size. r = 0.984, slope = 1.11 ± 0.07.

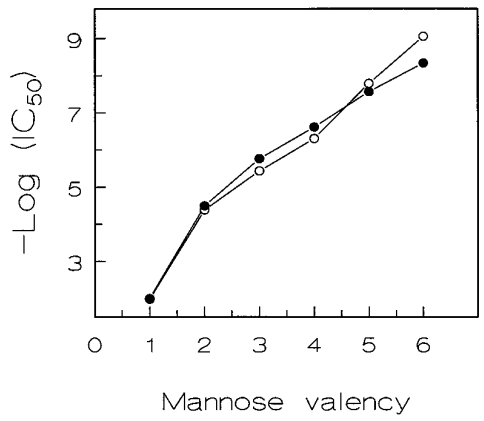


FIG. 5. **Effect of the mannose valency on the potency of cluster mannosides to inhibit binding of biotinylated ribonuclease B (●) or t-PA (○) to isolated human mannose receptor.** Inhibitory potencies of the cluster mannosides are derived from the data in Table I and are expressed as $-\log(IC_{50}) \pm$ S.D. Invisible error bars indicate errors smaller than the *symbol* size.

33% stimulation combined with a positive cooperative inhibition (n_H = 1.96 ± 0.143; data not shown). Since the stimulatory effect and the high apparent Hill coefficients were found in both receptor sorbent assays and in the endothelial cell binding assay (M_6L_5), we assume that it did not involve an artifact. Nonlinear regression analysis according to a two-site mixed stimulation/inhibition model showed that α-D-mannose induced a maximal stimulation of bio-Ribo B and bio-t-PA binding of 53 and 58%, respectively, at low concentrations. Half-maximal stimulation of ligand binding to the mannose receptor was attained at 250 μM ($-\log(SC_{50})$ = 3.6 ± 0.2) and 400 μM ($-\log(SC_{50})$ = 3.4 ± 0.2), respectively. Full inhibition of ligand binding was attained at a $-\log(IC_{50})$ of 2.00 ± 0.05 and 1.98 ± 0.04, respectively. The above mixed stimulation/inhibition model excellently predicted the observed steep inhibition curve for α-D-mannose.

DISCUSSION

In this study we have investigated the effect of the mannose valency of a cluster mannoside on its affinity for the mannose receptor. The ability of cluster mannosides to inhibit the binding of Ribo B, an established mannose receptor ligand (1), to the isolated human mannose receptor was determined in a receptor sorbent binding assay. Previous studies by Otter *et al*. (1, 9, 25) have established that this assay provides a reliable estimate of the affinity of monosaccharides, proteins with high mannose-type glycosides, and mannosylated neoglycoproteins for the mannose receptor. The validity of the receptor sorbent assay was further documented by the finding that the K_d for bio-Ribo-B (550 ± 70 nM) and bio-t-PA (1.66 ± 0.05 nM) in the receptor sorbent assay paralleled K_i values derived from competition studies of ^{125}I-t-PA binding to endothelial liver cells (190 ± 70 μM and 3.4 ± 0.4 nM, respectively).

The competition studies showed that all mannosides were able to inhibit bio-Ribo B binding to the isolated mannose receptor. The affinity of the synthesized mannosides was strongly and consistently enhanced with increasing mannose valency. Since the apparent inhibition constants $K_{i\ (app)}$ in the bio-Ribo B and bio-t-PA binding assays were essentially equal and ribonuclease B inhibits bio-t-PA binding in a competitive fashion, we may assume that inhibition of bio-Ribo B binding by mannosides reflects competition for ligand binding to the mannose receptor. In addition, it suggests that binding data from the bio-Ribo B assay are not significantly perturbed by heterogenicity of the sugar moiety from bio-Ribo B. The most complex mannosides from this study, M_5L_4 (**2d**) and M_6L_5 (**2e**) displayed nanomolar affinities for the mannose receptor that are at least comparable with those of mannosylated albumin (16), mannosylated poly-L-lysine (26, 27), or endogenous glycoproteins such as t-PA (9). Competition studies of ^{125}I-t-PA binding to endothelial liver cells confirmed the nanomolar affinity of M_6L_5. Previous efforts to synthesize low molecular weight ligands for the mannose receptor yielded compounds with 1000-fold lower, micromolar affinities for the mannose receptor (15, 28, 29). Oshimi *et al*. (28) demonstrated that the affinity of tris(hydroxymethyl)-based mannosides was only slightly higher than that of bi- and monosubstituted analogues, which was attributed to the suboptimal valency and spacing of the terminal mannosyl groups (~9 Å) within the mannose clusters. The di-, tri-, and tetramannosides synthesized by Robbins *et al*. (29) displayed higher, yet micromolar, affinities for the mannose receptor. Since the latter mannosides contained the same oligolysine backbone as our mannosides, their lower affinity is probably caused by differences in the chemical nature and the length of the spacers connecting the terminal

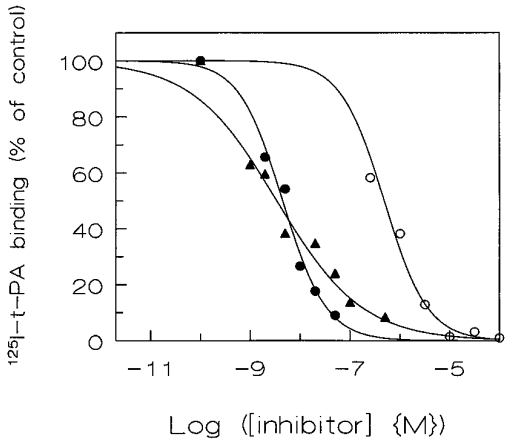


FIG. 6. **Competition studies of ^{125}I-t-PA binding to endothelial liver cells by bio-Ribo B (○), bio-t-PA (●), and M_6L_5 (▲).** Rat endothelial liver cells (2×10^6; 150 μg of cell protein) in DMEM (0.5 ml), containing 2% (w/v) BSA and ^{125}I-t-PA (1 nM), were incubated for 2 h at 4 °C with a variable concentration of displacer. Following incubation, cells were washed twice with DMEM, 0.2% BSA and once with DMEM, and cell-associated radioactivity was counted. Nonspecific binding is defined as ^{125}I-t-PA binding in the presence of 100 mM α-D-mannose.

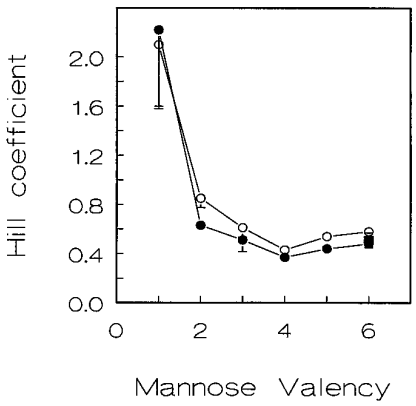


FIG. 7. **Effect of the valency of the (cluster) mannosides on the Hill coefficient of bio-Ribo B (●), bio-t-PA (○) and ^{125}I-t-PA (■).** Hill coefficients were calculated from the displacement curves in Figs. 3, 4 and 6 by nonlinear regression analysis as described under "Experimental Procedures."

α-D-mannose groups with the lysine backbone. In theory, the phenyl group of the (*p*-hydroxyanilino)carbamide spacer used in this study may contribute to ligand binding through π-π interaction forces (30). Since preliminary binding studies revealed that the affinity of 4-aminophenyl-α-D-mannopyranoside is only slightly higher than that of α-D-mannose ($K_{i\,(app)}$ = 1.8 mM, log(IC_{50}) = −2.48 ± 0.01; data not shown), we assume this to be unlikely. An alternative explanation is that the (*p*-hydroxyanilino)carbamide spacer is longer (2 Å) than the thioproprionyl spacer used by Robbins *et al*. (29). Recent studies by Biessen *et al*. (31) have already emphasized the relevance of optimal spacing of terminal glycosides to achieve avid recognition by a comparable eukaryotic lectin, the asialoglycoprotein receptor.

The $\Delta G^0_{(binding)}$ from M_2L, calculated from the logarithm of the $K_{i\,(app)}$, is almost twice as large as that for α-D-mannose (−24.4 kJ/mol and −13.3 kJ/mol, respectively). In the case of competitive inhibition, this suggests that both mannose groups from M_2L participate similarly in the binding process. The binding energy for M_6L_5 (−45 kJ/mol) is, in turn, 2-fold higher than that of M_2L, suggesting that four α-D-mannose groups from M_6L_5 interact independently with the mannose receptor. This seems to contrast with the finding that the affinity increases steadily rather than stepwise with increasing mannose valency. This apparent paradox may be explained from the negative cooperative binding of M_3L_2–M_6L_5; binding of an additional α-D-mannose to the receptor may attenuate the association of already bound mannose groups. Alternatively, the observed gain in binding energy with mannose valencies ≥2 may arise from conformational effects. In that case, elongation of the cluster mannoside with a single mannose group may force adjacent mannose groups into a geometric configuration favorable for binding to the mannose receptor.

The complex nature of ligand binding by the mannose receptor was further illustrated by the Hill coefficients of the inhibition curves for cluster mannosides. The Hill coefficient of α-D-mannose was significantly larger than 1. From the nonlinear regression analysis according to a two-site mixed stimulation/inhibition model, it can be concluded that the high Hill coefficient reflects a compromise between a stimulatory effect of α-D-mannose at low concentrations (SC_{50} = 250–400 μM) and an inhibitory effect of α-D-mannose at an IC_{50} of 10 mM. Binding of the first α-D-mannose group to the high affinity site of the mannose receptor (possibly CRD4) may result in a 2-fold increased affinity of bio-t-PA or bio-Ribo B for the mannose receptor as was also reported by Oshimi *et al.* (28). In agreement with Oshimi *et al*. (28), this stimulatory effect is less pronounced for moderately potent ligands like M_2L and absent for potent ligands like M_3L_2 to M_6L_5, as was reported in the same study (28). At higher concentrations, bound ligand is displaced from the mannose receptor by binding of α-D-mannose to the low affinity site.

By contrast, M_3L_2 to M_6L_5 gave Hill coefficients of about 0.5 in both receptor sorbent assays and in the endothelial cell assay (M_6L_5), while Hill coefficients of bio-Ribo B or bio-t-PA indicated binding to a single site. Taking into account that the mannose receptor is a monomeric membrane-bound protein, this concurs with ligand binding to two (or more) separate but interacting sites within the mannose receptor. According to the binding model of Taylor and Drickamer *et al*. (14), high affinity binding of proteins with high mannose-type glycosides like t-PA or ribonuclease B requires the cooperative action of CRD4 and CRD5. For optimal recognition of highly mannosylated polymers like mannan and invertase, the presence of five consecutive CRDs (CRD4–8) is imperative. Taken together, it is tempting to identify CRD4 in concert with CRD5 as the first and CRD6–8 as the second binding unit. The first unit, embedding two carbohydrate recognition domains, is responsible for the binding of bio-Ribo B, bio-t-PA, α-D-mannose, and M_2L. For binding of polyvalent mannosides like M_3L_2 to M_6L_5 and mannan, both the first (CRD4–5) and the second unit (CRD6–8) participate in the binding process. Nonetheless, further study will be needed to conclusively unravel the intriguing process of ligand binding to the mannose receptor.

In conclusion, we have devised and synthesized high affinity ligands for the mannose receptor, M_6L_5 being the most potent ligand yet synthesized. We anticipate that the nanomolar affinity of M_6L_5 is sufficiently high to allow application as a carrier device for cell-specific delivery of drugs/genes to macrophages and endothelial liver cells. Another relevant application that comes within reach involves antigen targeting to dendritic cells to stimulate major histocompatability complex-mediated antigen presentation by this cell type. Finally, M_6L_5 may also be a valuable therapeutic agent to prevent untimely clearance of the thrombolytic agent t-PA. In fact, preliminary studies in rats showed that administration of this mannoside resulted in a 2-fold delayed clearance of t-PA (32).

REFERENCES

1. Maynard, Y., and Baenziger, J. U. (1981) *J. Biol. Chem.* **256,** 8063–8068
2. Lennartz, M. R., Cole, F. S., Shepherd, V. L., Wileman, T. E., and Stahl, P. D.

(1987) *J. Biol. Chem.* **262,** 9942–9944
3. Stahl, P. D. (1990) *Am. J. Respir. Cell. Mol. Biol.* **2,** 317–318
4. Otter, M., Kuiper, J., van Berkel, T. J. C., and Rijken, D. C. (1992) *Ann. N.Y. Acad. Sci.* **667,** 431–442
5. Ezekowitz, R. A. B., Williams, D. J., Koziel, H., Armstrong, M., Warner, A., Richards, F. F., and Rose, R. M. (1991) *Nature* **351,** 155–158
6. Brunda, M. J., Wiltrout, R. H., Holden, H. T., and Varesio, L. (1983) *Int. J. Cancer* **31,** 373–379
7. Ezekowitz, R. A. B., Sastry, K., Bailly, P., and Warner, A. (1990) *J. Exp. Med.* **172,** 1785–1794
8. Smedsrød, B., Melkko, J., Ristelli, L. and Ristelli, J. (1991) *Biochem. J.* **271,** 345–350
9. Otter, M., Barrett-Bergshoeff, M. M., and Rijken, D. C. (1991) *J. Biol. Chem.* **266,** 13931–13935
10. Stahl, P. D., and Schlesinger, P. H. (1980) *Trends Biochem. Sci.* **5,** 194–195
11. Malhotra, R., Wormald, M. R., Rudd, P. M., Fischer, P. B., Dwek, R. A., and Sijm, R. B. (1995) *Nat. Med.* **1,** 237–244
12. Taylor, M. E., Conary, J. T., Lennartz, M. R., Stahl, P. D., and Drickamer, K. (1990) *J. Biol. Chem.* **265,** 12156–12162
13. Taylor, M. E. (1993) *Biochem. Soc. Trans.* **21,** 468–473
14. Taylor, M. E., and Drickamer, K. (1993) *J. Biol. Chem.* **268,** 399–404
15. Kéry, V., Křepinskí, J. J. F., Warren, C. D., Capek, P., and Stahl, P. D. (1992) *Arch. Biochem. Biophys.* **298,** 49–55
16. Hoppe, C. A., and Lee, Y. C. (1983) *J. Biol. Chem.* **258,** 14193–14199
17. Jansen, R. W., Molema, G., Ching, T. L., Oosting, R., Harms, G., Moolenaar, F., Hardonk, M. J., and Meijer, D. K. F. (1991) *J. Biol. Chem.* **266,** 3343–3348
18. Monsigny, M., Kieda, C., and Roche, A. (1983) *Biol. Cell* **47,** 95–110
19. Kataoka, M., and Tavassoli, M. (1984) *J. Histochem. Cytochem.* **32,** 1091–1098
20. Fraker, P. J., and Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80,** 849–857
21. De Rijke, Y. B., Jurgens, G., Hessels, E. M. A. J., Hermann, A., and Van Berkel, T. J. C. (1992) *J. Lipid Res.* **33,** 1315–1325
22. Otter, M., Zockova, P., Kuiper, J., Van Berkel, T. J. C., Barrett-Bergshoeff, M. M., and Rijken, D. C. (1992) *Hepatology* **16,** 54–59
23. Biessen, E. A. L., Bakkeren, H. F., Beuting, D. M., Kuiper, J., and Van Berkel, T. J. C. (1994) *Biochem. J.* **299,** 291–296
24. Biessen, E. A. L., Norder, J. A., Horn, A. S., and Robillard, G. T. (1988) *Biochem. Pharmacol.* **37,** 3959–3966
25. Otter, M., Kuiper, J., Bos, R., Rijken, D. C., and Van Berkel, T. J. C. (1992) *Biochem. J.* **284,** 545–551
26. Hoppe, C. A., and Lee, Y. C. (1984) *Biochemistry* **23,** 1723–1730
27. Derrien, D., Midoux, P., Petit, C., Negre, E., Mayer, R., Monsigny, M., and Roche, A. C. (1989) *Glycoconjugate J.* **6,** 241–255
28. Oshimi Y., Hoppe, C. A., Ogawa, T., and Lee, Y. C. (1988) *Arch. Biochem. Biophys.* **260,** 241–249
29. Robbins, J. C., Hanh Lam, M., Tripp, C. S., Bugianesi, R. L., Ponpipom, M. M., and Shen, T. Y. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7294–7298
30. Lee, R. T., Ichikawa, Y., Fay, M., Drickamer, K., Shao, M.-C., and Lee, Y. C. (1991) *J. Biol. Chem.* **266,** 4810–4815
31. Biessen, E. A. L., Beuting, D. M., Roelen, H. C. P. F., Van de Marel, G. A., Van Boom, J. H., and Van Berkel, T. J. C. (1995) *J. Med. Chem.* **38,** 1538–1546
32. Biessen, E. A. L., Kuiper, J., Van Teijlingen, M. E., Vietsch, H., Bijsterbosch, M. K., Rijken, D. C., and Van Berkel, T. J. C. (1996) *Circulation* **92,** I692
33. Taylor, M. E., Bezouöka, K., and Drickamer, K. (1992) *J. Biol. Chem.* **267,** 1719–1726