Glycoconjugate Journal (1995) **12**: 275–281

Versatile synthesis of bi- and tri-antennary galactose ligands: interaction with the Gal/GalNAc receptor of human hepatoma cells

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Received 19 September 1994, revised 15 December 1994

We have synthesized bi- and tri-antennary galactose ligands by coupling 1-thio-β-D-galactose derivatives to the α- and ε-amino groups of L-lysine and L-lysyl-L-lysine via highly flexible hydrophilic spacer arms that allow variation of their intergalactose distances. The interaction of these ligands with the Gal/GalNAc receptor of HepG2 cells showed a binding affinity that was: (i) in agreement with the clustering effect known to occur with more complex oligomeric structures, i.e. tri- > bi-antennary; ii) dependent on the intergalactose distances (optimal interactions were observed for the tri-antennary structures with distances > 2 nm). These ligands, that can be easily conjugated to bioactive (macro) molecule carrier systems, could be useful for their targeting to hepatocytes.

Keywords: HepG2 cells, hepatocytes, asialoglycoprotein receptor, cluster galactosyl glycosides, targeting

Introduction

Receptors expressed at the surface of cells, that mediate endocytosis of their ligands, are attractive targets for delivering bioactive (macro) molecules to specific cells. The Gal/GalNAc receptor present at the surface of mammalian hepatocytes (i.e. the asialoglycoprotein receptor), has been well characterized at both the physiological and molecular level [1, 2] and was used for the targeting, *in vitro* and *in vivo*, of drug carriers such as liposomes [3, 4], neogalactoproteins [5] or poly(L-lysine)-based gene delivery systems [6, 7]. A lectin that similarly binds galactosyl ligands was also found at the surface of macrophages [8, 9] and certain metastases [10].

The ligands associated with such delivery systems range from glycoproteins, such as asialoorosomucoid (ASOR) or asialofetuin [3, 7], to synthetic molecules [4, 6, 11]. Efficient binding of galactosyl ligands to the Gal/GalNAc receptor is dependent on several factors which take into account the particularities of this lectin. Thus, the highest affinities were measured for oligomeric structures such as tri- and tetra-galactosyl ligands (synthetic glycosides or isolated multi-antennary glycopeptides) [12, 13]. Moreover, optimal interactions with the clustered galactosides ('cluster effect') also require well defined inter-galactose distances [14] and geometries [15]. Recently we have described chemical strategies to prepare liposomes bearing at their surface mono- [16] and tri-antennary [17] galactosyl ligands and studied their interaction with mouse resident peritoneal macrophages. In the present study we have further extended this strategy which, because of its flexibility, allows the preparation of multi-antennary galactosyl ligands, differing in their inter-galactose distances, and which can be easily conjugated to drug carriers or gene delivery systems. The ligands were compared for their affinity for the Gal/GalNAc receptor expressed at the surface of HepG2 cells [18], a human hepatoma cell line.

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Materials and methods

Synthesis

L-Lysine, HCl and L-lysyl-L-lysine, 2HCl (Serva) were converted into their trifluoroacetate salts after dissolution in trifluoroacetic acid at 35°C and precipitation into diethyl ether [19]. For the detection of carbohydrates, TLC plates (60-F_{254};Merck) were sprayed with a solution of α-naphtol in sulfuric acid:ethanol (1:9) and heated to 120°C. Amino groups were detected by spraying with a solution of 0.2% ninhydrin in ethanol and heating the plates briefly. Dimethylformamide was redistilled over ninhydrin and methylene chloride over CaH_2 before use. ^{1}H-NMR spectra were recorded on a Bruker WP-200 MHz SY spectrometer (chemical shifts are given in ppm and the solvents used were: $CDCl_3$ at 7.27; D_2O with t-BuOH at 1.28 ppm). Electrospray ionization (ESI)-mass spec-

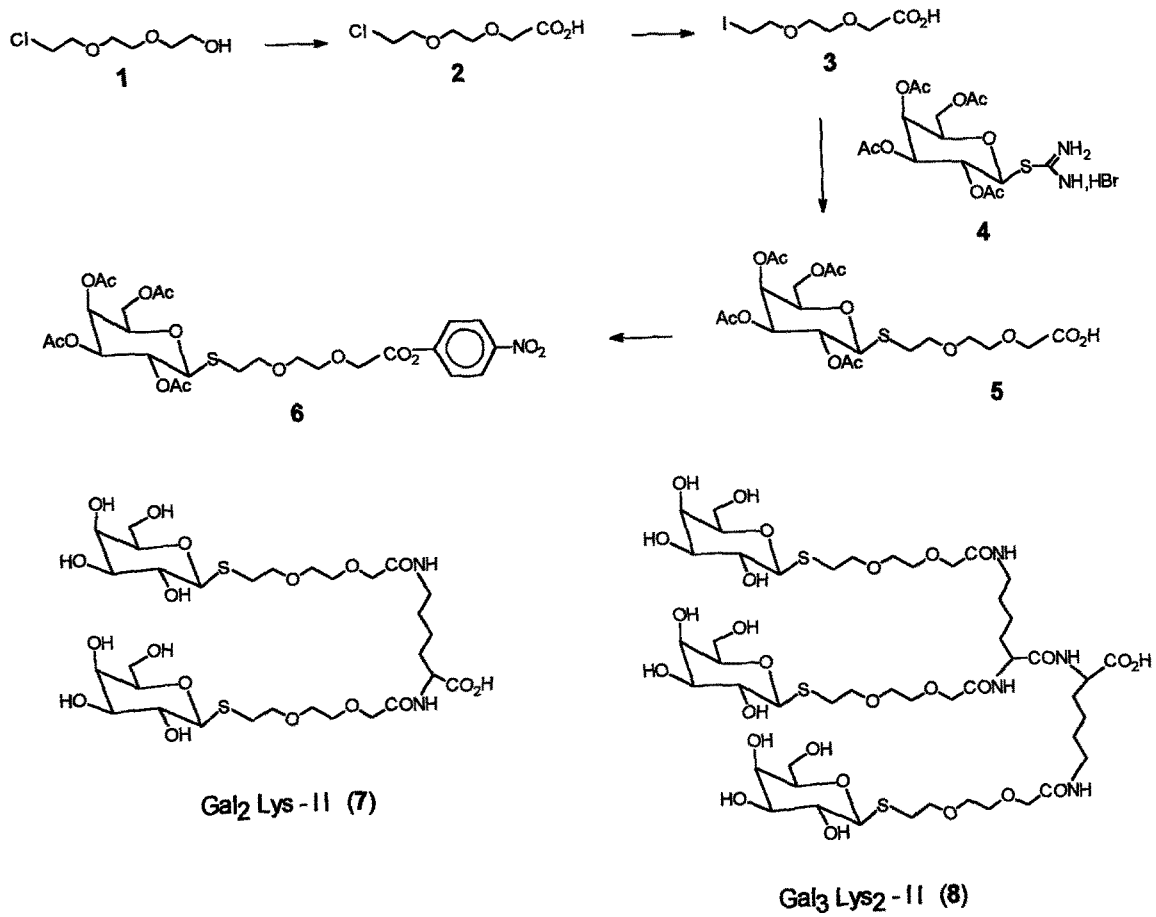

Figure 1. Structure of the multi-antennary Gal_2Lys-II (**7**) and Gal_3Lys_2-II (**8**) ligands and synthesis of the intermediate (**6**).

trometry was performed on a Bio-Q VG BioTech (Altrincham, UK) apparatus.

Synthesis of Gal_2Lys-II (7) and Gal_3Lys_2-II (8) (Fig. 1)

2-(2-(2-chloroethoxy)ethoxy)ethanoic acid (2) Compound **1** (4g, 23.6 mmol; Aldrich) dissolved in 300 ml acetone was treated dropwise over 2 h, while stirring, with 12 ml of 2.67 M Jones reagent (prepared by dissolving 4 g of chromic trioxide in 3.45 ml of concentrated sulfuric acid and diluted to a volume of 15 ml). After 1 more h, a few drops of 2-propanol were added, and 20 min later 200 ml water were poured in to dissolve the precipitated chromium salts, and the solvent was removed under reduced pressure. The aqueous mixture, to which 100 ml of saturated NaCl solution were added, was extracted with 6×100 ml CH_2Cl_2. The combined extracts, dried over $MgCl_2$ and filtered, were evaporated to dryness. Product **2** was isolated, after purification by chromatography on a Dowex 1X8 (OH^-) column eluted with 1 N HCl in 44% yield as a yellow oil. It gave a single spot on TLC: $R_F = 0.46$ (CH_2Cl_2:MeOH:AcOH. 25: 1: 1).

^{1}H-NMR (200 MHz; $CDCl_3$) δ: 3.64 (t, 2H, $ClCH_2$), 3.74–3.78 (m, 6H, CH_2O), 4.20 (s, 2H, CH_2COOH), 8.03 (bs, 1H, COO*H*).

2-(2-(2-iodoethoxy)ethoxy)ethanoic acid (3) A solution of **2** (1.93 g, 10.55 mmol) and sodium iodide (3.15 g, 21 mmol) in 45 ml of 2-butanone was heated under reflux for 20 h. The mixture was then filtered to remove the salts and, after evaporation of the solvent, the residue was dissolved in 20 ml methylene chloride. The resulting solution was treated with 6 ml $NaHSO_3$ (37.5% in water), followed by three washings with 20 ml portions of a saturated NaCl solution. The product **3** (yield 72%), a viscous liquid, was used without further purification.

(2-(2-(2,3,4,6-Tetra-O-acetyl-1-thio-β-D-galactopyranosyl) ethoxy) ethoxy) acetic acid (5) A mixture consisting of **3** (2.1 g, 7.6 mmol), **4** (3.65 g, 7.5 mmol) [16], sodium carbonate (0.89 g, 8 mmol), and sodium metabisulfite (1.52 g, 8 mmol) in 16 ml acetone:water (1:1, v:v) was stirred at room temperature. After 3 h, the mixture was acidified with 7.5 ml HCl (5%), and extracted four times with ethyl acetate. The oily reaction product **5** was purified on a column of silica gel eluted with CH_2Cl_2:EtOH:AcOH, 30:0.5:0.2 (yield 65%). It gave a single spot on TLC with a $R_F = 0.35$ (CH_2Cl_2:EtOH:AcOH, 30:1:1).

^{1}H-NMR (200 MHz; $CDCl_3$)δ: 1.98, 2.05, 2.09 and 2.15 (4s, each 3H, $4COCH_3$), 2.9 (m, 2H, SCH_2), 3.65–3.77 (m, 6H, $3OCH_2$), 3.97– 4.16 (m, 5H, H-5, H-6′ H-6″, OCH_2CO),

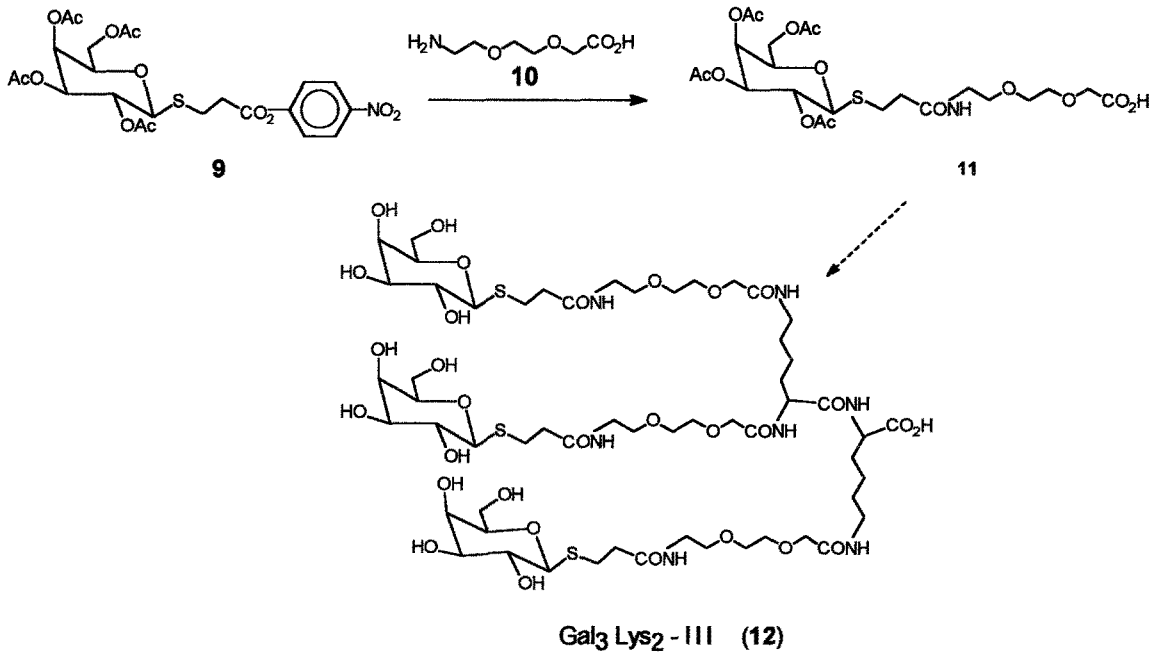

Figure 2. Synthesis of Gal_3Lys_2-III (**12**).

4.60 (d, 1H, $J_{1,2}$ 9.6 Hz, β-configuration, H-1), 5.07 (dd, 1H, $J_{2,3}$ 9.9 Hz, $J_{3,4}$ 3.2 Hz, H-3), 5.26 (dd, 1H, H-2), 5.41 (d, 1H, $J_{3,4}$ 3.1 Hz, H-4), 8.2 (bs, COOH).

MS (ESI-): *m/z*=509.1 $(M-H)^{1-}$ (510.14, calcd for $C_{20}H_{30}O_{13}S$).

(2-(2-(2,3,4,6-Tetra-O-acetyl-1-thio-β-D-galactopyranosyl) ethoxy)ethoxy)acetic acid 4-nitrophenyl ester (6) For the coupling of compound **5** to lysine and dilysine trifluoroacetate salts, its carboxylate function was first activated as a *p*-nitrophenyl ester [19]. Briefly, to **5** (3.15 g; 5 mmol) in dry methylene chloride (20 ml), were added *p*-nitrophenol (0.97 g; 7 mmol) and N,N′-dicyclohexylcarbodiimide (DCC) (1.34 g; 6.5 mmol). After 4 h stirring at room temperature under argon, and removal of the N,N′-dicyclohexylurea (DCU) precipitate by filtration, the reaction product **6**, a viscous liquid, was purified on a silica gel column eluted with CH_2Cl_2:EtOH: AcOH, 30:1:0.2 (50% yield). R_F(TLC)=0.22 (CH_2Cl_2:AcOH, 30: 1)

^{1}H-NMR (200 MHz; $CDCl_3$) δ: 1.97, 2.01, 2.05 and 2.13 (4s, each 3H, $4COCH_3$), 2.75–3.06 (m, 2H, SCH_2), 3.67–3.84 (m, 6H, $3OCH_2$), 3.95–4.2 (m, 3H, H-5, H-6′, H-6″), 4.45 (s, 2H, CH_2CO), 4.63 (d, 1H, $J_{1,2}$ 9.8 Hz, H-1), 5.03 (dd, 1H, *J* 9.8 and 3.3 Hz, H-3), 5.2 (t, 1H, H-2), 5.40 (d, 1H, *J* 2.75 Hz, H-4), 7.32 (d, 2H, 2 × (*CH*)CO), 8.27 (d, 2H, 2(*CH*)CNO_2)).

Gal_2Lys-II (7) This was obtained by adding 1.3 mmol of **6** to L-lysine, trifluoroacetate (0.13 g; 0.5 mmol) in 10 ml DMF. After 17 h at room temperature, the solvent was evaporated. Without further purification, the reaction product was deacetylated in 10 ml of MeOH:H_2O:diisopropylethylamine (DIPEA), 5: 4: 1. After 72 h of stirring, at room temperature, the deprotection was complete and the solvents were evaporated under reduced pressure to dryness. The residue was purified on a column of silica gel eluted with $CHCl_3$:MeOH:H_2O; 60:30:5. A white solid was obtained (yield over the two steps: 68%). R_F (TLC) = 0.22 ($CHCl_3$:MeOH:H_2O; 60:40:10).

^{1}H-NMR (200 MHz; D_2O) δ: 1.25–1.90 (m, 6H, $3CH_2$ of lysine), 2.96 (m, 4H, $2SCH_2$), 3.23 (t, 2H, *J* 6.6 Hz, CH_2NH), 3.48 – 3.6 (m, 22H, 6(CH_2O), 2(H-2, H-3, H-5, H-6′, H-6″), 3.94 (d, 2H, *J* 2.6 Hz, H-4), 4.04 (s, 2H, OCH_2CONH), 4.08 (s, 2H, OCH_2CONH), 4.2 (m, 1H, HNC*H*CO), 4.48 (d, 2H, $J_{1,2}$ 9.25 Hz, 2H-1).

MS(ESIFI): *m/z* = 793.3 $(M-H)^{1-}$ (794.28, calcd for $C_{30}H_{54}N_2O_{18}S_2$).

Gal_3Lys_2-II (8) Compound **6** (2g; 3 mmol) was added to a solution of L-lysyl-L-lysine, trifluoroacetate (0.45 g; 0.9 mmol) in 20 ml DMF containing DIPEA (0.605 ml, 3.5 mmol). The mixture was stirred for 48 h at room temperature under argon and then evaporated to dryness. The reaction product was deacetylated as above (48 h). Compound **8**, a white solid, was purified on a silica gel column eluted with $CHCl_3$:MeOH:H_2O; 60:50:16 (yield over the two steps: 49%). R_F (TLC) = 0.31 ($CHCl_3$:MeOH:H_2O; 60:50:16).

^{1}H-NMR (200 MHz; D_2O) δ: 1.22–1.87 (m, 12H, $6CH_2$ dilysine), 2.87–3.06 (m, 6H, $3SCH_2$, 3.22 (m, 4H, 2 × CH_2NHCO), 3.57–3.80 (m, 33H, 3(H-2, H-3, H-5, H-6′, H-6″, $9CH_2O$), 3.95 (d, 3H, 3H-4), 4.05 (s, 4H, $2OCH_2CONH$),

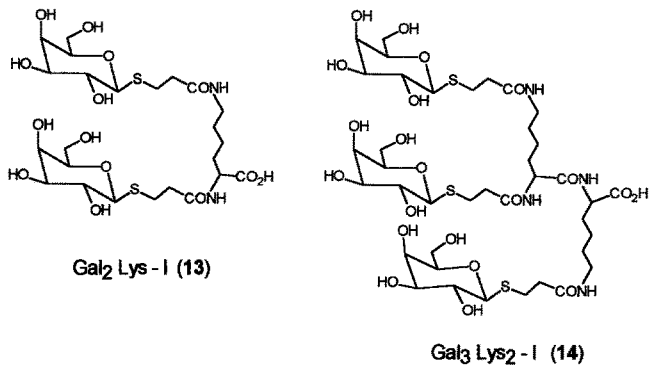

Figure 3. Structure of the multi-antennary Gal_2Lys-I (**13**) and Gal_3Lys_2-I (**14**) ligands.

4.12 (s, 2H, OCH_2CONH), 4.18 (m, 1H, NHC*H*CO), 4.31–4.42 (m, 1H, NHC*H*CO), 4.48 (d, 3H, $J_{1,2}$ 9.2 Hz, 3H-1).

MS(ESI-): *m/z*=1245.9 $(M-H)^{1-}$, *m/z*=622.6 $(M-2H)^{2-}$ (1246.46 calcd for $C_{48}H_{86}N_4O_{27}S_3$).

*Synthesis of Gal3Lys2-III (**12**; Fig. 2)*

Compound **11** was obtained by adding the trifluoroacetate salt of the amino acid 10 [20] (1.6 g; 10 mmol) to 90 ml of DMF containing 10 mmol of p-nitrophenyl 3-(2, 3, 4, 6-tetra-O-acetyl-1-thio-β-D-galactopyranosyl)propionate (**9**) [16, 19] and 15 mmol (2.6 ml) of DIPEA. After 72 h, at room tempera ture under argon, the mixture was evaporated under vac uum. The oily reaction product was purified on a column of silica gel eluted with AcOEt:AcOH:EtOH,25: 5:3(yield 62 %) R_F (TLC) = 0.36(AcOEt:EtOH;25: 7:3).

^{1}H-NMR (200 MHz; $CDCl_3$)δ: 1.95, 2.01, 2.03 and 2.13 (4s, each 3H, $4COCH_3$), 2.45–2.65 (m, 2H, SCH_2CH_2), 2.99 (m, 2H, SCH_2), 3.46 – 3.67 (m, 8H, $NHCH_2$, $3OCH_2$), 3.98–4.16 (m, 5H, H-5, H-6′, H-6″, OCH_2COOH), 4.55 (d, 1H, $J_{1,2}$ 9.5 Hz, H-1), 5.06 (dd, 1H, $J_{2,3}$ 9.7 Hz, $J_{3,4}$ 2.3 Hz, H-3), 5.16 (t, 1H, $J_{1,2}$ = $J_{2,3}$ 9.9 Hz, H-2), 5.41 (d, 1H, $J_{3,4}$ 2.4 Hz, H-4), 9.58 (bs,COOH).

MS(ESI-): *m/z*=580.1 $(M-H)^{1-}$(581.17, calcd for $C_{23}H_{35}NO_{14}S$).

Compound **12** was obtained in 31% yield by coupling the *p*-nitrophenyl ester of **11** (R_F (TLC) = 0.54 (AcOEt: AcOH:EtOH, 25:2:1; yield 40%)) with dilysine and de-acetylation as described above for the preparation of **8**. Gal_3Lys_2-III was purified on a silica gel column eluted with $CHCl_3$:MeOH:H_2O, 60:40:10. R_F(TLC) = 0.44 ($CHCl_3$: MeOH:H_2O; 60:40:10)

^{1}H-NMR (200 MHz; D_2O) δ: 1.19 – 1.93 (m, 12H, $6CH_2$ of dilysine), 2.60 (t, 6H, $3SCH_2CH_2CONH$), 2.92–3.01 (m, 6H, $3SCH_2$), 3.22 (m, 4H, 2ε-CH_2), 3.39 (t, 6H, *J* 5.2 Hz, $3CONHCH_2CH_2O$), 3.56–3.75 (m, 33H, 3(H-2, H-3, H-5, H-6′ and H-6″), $6CH_2O$ and $3OCH_2$), 3.94 (d, 3H, 3H-4), 4.04 (s, 4H, $2OCH_2CONH$), 4.11 (s, 2H, OCH_2CONH), 4.15 (m, 1H, NHC*H*CO), 4.3– 4.4 (m, 1H, NHC*H*CO), 4.45 (d, 3H, $J_{1,2}$ 9.3 Hz, 3 H-1).

MS (ESI+) *m/z* = 730.9 $(M+2H)^{2+}$; 741.7 $(M+Na+H)^{2+}$; 753.2 $(M+2Na)^{2+}$ (1458.56, calcd for $C_{57}H_{100}N_7O_{30}S_3$).

*Synthesis of Gal_2Lys-I (**13**) (Fig. 3)*

Compound **9** (0.9 g; 1.6 mmol) was added to a solution of L-lysine, trifluoroacetate salt (0.209 g; 0.8 mmol) in DMF (8 ml) and triethylamine (0.351 ml; 2.6 mmol). After 18 h at room temperature, under argon, the solvents were evaporated and the reaction product purified by chromatography on a silica gel column eluted with $CHCl_3$:MeOH:H_2O, 90:10:1. A white solid was obtained in 50% yield. R_F(TLC) = 0.74 ($CHCl_3$:MeOH:H_2O, 60:40:10). Compound **13** was obtained, in 75% yield, after deacetylation as described for the preparation of **8** and **12** (see above) and chromatography on a silica gel column eluted with $CHCl_3$:MeOH:H_2O, 60:40:10. R_F (TLC) = 0.13 ($CHCl_3$:MeOH:H_2O, 60:40:10).

^{1}H-NMR (200 MHz; D_2O)δ: 1.35–1.85 (m, 6H, $3CH_2$ of lysine), 2.66 (m, 4H, $2CH_2CONH$), 3.03 (m, 4H, $2SCH_2$), 3.22 (t, 2H, *J* 6.5 Hz, ε-CH_2 of lysine), 3.53–3.82 (m, 10 H, 2(H-2, H-3, H-5, H-6′, H-6″)), 4.0 (d, 2H, *J* 2.9 Hz, 2H-4), 4.17 (q, 1H, NHC*H*CO), 4.51 (d, 1H, $J_{1,2}$ 9.3 Hz, H-1), 4.53 (d, 1H, $J_{1,2}$ 9.3 Hz, H-1).

MS (ESI-): *m/z*=645 $(M-H)^{1-}$ (646.2, calcd for $C_{24}H_{42}N_2O_{14}S_2$).

Cell culture

The human hepatoma HepG2 cell line (from ATCC) was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco), 1g l^{-1} glucose, 50 U ml^{-1} penicillin G and 50 μg ml^{-1} streptomycin sulfate. Hepatocytes were prepared from male Wistar rats (average 250 g) by the collagenase perfusion procedure [21]. Briefly, the liver was perfused, at 39°C, through the portal vein, with 200 ml (30 ml min^{-1}) 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)/phosphate buffered saline (NaCl 140 mM; KCl 5 mM; Na_2HPO_4 1mM; Hepes 10 mM; pH 7.6) followed by 250 ml (15 ml min^{-1}) Hepes buffered saline (HBS) containing 25 mg per 100 ml collagenase (type D; Boehringer Mannheim) and 75 mg per 100 ml $CaCl_2$. The liver was then dissociated and the suspension filtered through a 100 μm nylon sieve. Hepatocytes were obtained by brief centrifugation (200 × **g**) in DMEM.

Preparation of [^{125}I] asioloorosomucoid

Human plasma orosomucoid (Sigma) was desialylated by mild hydrolysis in 0.025 m H_2SO_4, at 80°C for 1 h [22], and purified by gel filtration (M_r 43 kDa) on a Sephadex G-100 column (Pharmacia) equilibrated with phosphate buffered saline (PBS). The removal of sialic acid was essentially complete as measured by the thiobarbituric acid assay [22]. ^{125}I-ASOR was prepared using Na^{125}I (carrier free, Amersham) and the iodination reagent IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) from Pierce, according to Fraker

and Speck [23]. The labelled protein (average specific activity: 107 dpm per μg ASOR) was separated from $^{125}I_2$ by gel filtration on Sephadex G-25 eluted with PBS. When necessary, the specific activity of ^{125}I-ASOR was adjusted by dilution with unlabelled ASOR.

Binding assays

The binding affinities of the synthetic molecules were determined according to a competition assay. Multi-well (35 mm diameter) tissue culture plates (Nunc) were seeded, generally 4 days prior to a binding experiment, with $3–5 \times 10^5$ cells per well. At the time of assay, the HepG2 cells had grown to near confluence (about 1.5×10^6 cells). Cells were washed (2 ml) for 30 s, at room temperature, with PBS, containing 5 mM EDTA (pH 5.22), followed by DMEM (pH 7.4). To the monolayers, after a preincubation for 30 min at 4°C in DMEM containing 1 mg ml^{-1} cytochrome *c* and 10 mM Hepes, were added 7×10^{-10} M of ^{125}I-ASOR (specific activity: $2–5 \times 10^6$ cpm/per μg ^{125}I-ASOR) and increasing concentrations of the competing ligand (eight data points). After 2 h of incubation, at the same temperature, the medium was removed and the cells were washed three times with ice-cold HBS (50 mM Hepes, 141 mM NaCl and 3.8 mM KCl) containing 2.5 mM $CaCl_2$ to remove unbound label. The nonspecific binding was determined in each experiment by washing the cells for 3 min with 5 mM EDTA/PBS (pH 5.22), which abolishes the Ca^{2+}-dependent specific binding. The cells were then dissolved in 1 ml 0.1 N NaOH for 15 min at room temperature. Aliquots of 0.8 ml were counted in a gamma counter (LKB) and 50 μl were used for protein concentration determination, using a bicinchoninic acid (BCA)-based assay. The experiments with the rat hepatocytes were similarly done with about 2.5×10^6 cells per dish. During the 2 h incubation period with the ligands, the hepatocytes were gently swirled every 15 min and were finally collected by centrifugation and washed three times with 3 ml of HBS-2.5 mM $CaCl_2$. All binding assays were performed in triplicate and the results were expressed as radioactivity with respect to cellular protein. For each synthetic ligand tested,the value of I_{50} (i.e. the concentration required to inhibit 50% of the specific binding of ^{125}I-ASOR) was determined from the displacement curves (single binding site model) by use of a non-linear regression computer program (GraphPad, ISI Software). The standard deviation ($n = 3$) was less than 4%. The affinity of ^{125}I-ASOR for the HepG2 cells and rat hepatocytes was determined as a control; it also allowed the transformation of the I_{50} values into K_i by use of the Cheng-Prusoff equation [24].

Results and discussion

The aim of the present study was to produce simplified multi-antennary galactosyl structures possessing:(i) some of the key features that make more complex natural oligosaccharides, present e.g. in glycoproteins, potent and specific ligands for the Gal/GalNAc receptor; and (ii) a chemical function enabling them to be covalently conjugated to targeted bioactive (macro)molecule carrier systems. The ligand affinity of the galactose receptor is determined by the number of exposed terminal non-reducing D-galactose units [25], and by their distances within a cluster. Thus, for a tri-antennary oligosaccharide, an optimal binding for hepatocyte receptor was attained when the galactose moieties were at the apexes of a triangle of 1.5, 2.2 and 2.5 nm sides [14]. Moreover, in addition to these inter-galactose distances, it seems that a correct orientation of one residue with respect to the others is also of importance [15].

We have synthesized bi- and tri-antennary ligands that differ in their inter-galactose distances and are characterized by highly flexible structures (Figs 1–3); it was hoped that in such molecules the galactose residues could easily meet the spatial arrangement needed for optimal binding to the receptor. To that end we have linked galactosyl moieties to the α and ε-amino functions of lys or lys-lys, a strategy already explored by us [17] and others [19], via hydrophilic spacer-arms of variable lengths. This approach is versatile, i.e. it can be easily extended to a variety of spacer arms. Relatively few simplified multi-antennary galactosyl molecules have been made [6, 11, 17, 19, 26, 27] and, if successful, this approach of relatively small molecular weight ligands should find applications e.g. targeting to hepatocytes. In this respect, the presence in the ligands we have synthesized of a carboxylic function, distal from the galactose moieties, is destined to their bioconjugation (see [17]).

Chemistry The synthesis and use of hydrophilic polyoxyethylene-based spacer-arms **3** and **10** is shown in Figs. 1 and 2. Compound **3** was obtained from commercially available **1** by successive oxidation of the alcohol function with Jones reagent into acid (→**2**) and change of the chloro substituent into the more reactive iodo (→ **3**). After base-catalysed deprotection of the thiol function of the readily prepared [16] galactosyl derivative **4**, reaction with the bifunctional spacer-arm **3** afforded **5**. The choice of β-S-galactosyl derivatives resides in the observation that these compounds are more resistant towards hydrolysis, both chemically and enzymatically, than their corresponding *O*-derivatives without altering their interaction with the galactose receptor [4]. The carboxylic function of **5** was classically activated by formation of a *p*-nitrophenyl ester (→ **6**). Compound **6** was then reacted with the trifluoroacetates of L-lysine or L-lysyl-L-lysine, in DMF, to yield after removal of the acetate protective groups, respectively Gal_2Lys-II (**7**) and Gal_3Lys_2-II (**8**). The deprotection was best acheived in a water/methanol mixture in the presence of diisopropylethylamine.

Gal_3Lys_2-III (**12**) was prepared as described in Fig. 2. Briefly, compound **9**, synthesized as described previously [16, 19], was reacted in DMF with the trifluoroacetate of the amino acid **10**, prepared from **1** according to Slama and Rando [20]. The reaction product **11**, compared to **5**, has a longer spacer-

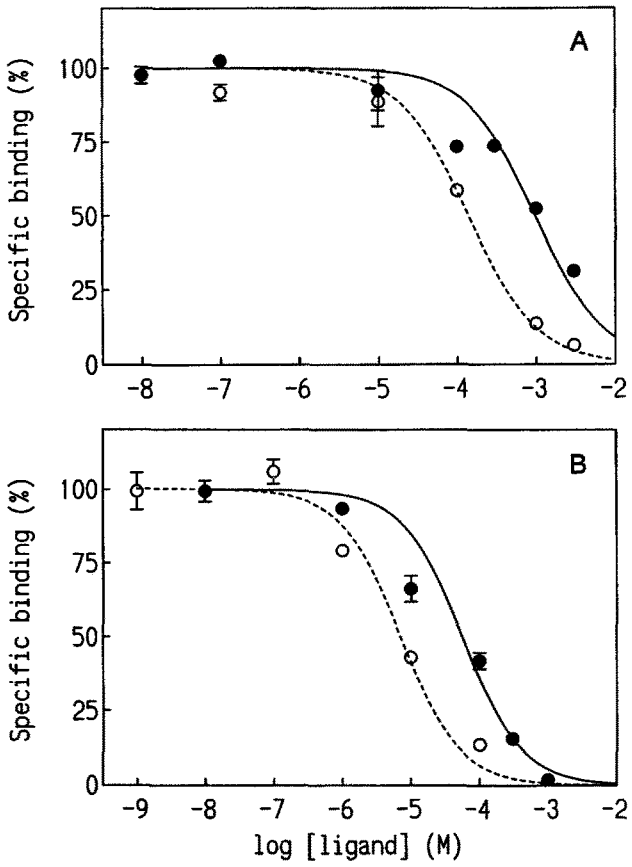

Figure 4. Semilog representation of the inhibition of ^{125}I-ASOR binding to the Gal/GalNAc receptor of (**A**) HepG2 cells and (**B**) rat hepatocytes by synthetic tri-antennary galactosyl ligands. Binding experiments (triplicates) were performed in 35 mm diameter culture dishes, for 2 h at 4°C, with HepG2 cells at confluency (1.5×10^6 cells) or freshly isolated rat hepatocytes (2.5×10^6 cells), using a fixed concentration of labelled ASOR (7×10^{-10} M) respectively in the absence or presence of increasing concentrations of the synthetic ligands. Binding is plotted as the percentage of the specific binding observed in the absence of a competing ligand. The curves were computer-generated by fitting the experimental data to a theoretical displacement curve (see Text). The following ligands were tested: Gal_3Lys_2-I (**14**) (—●—)(panel **A** and **B**); Gal_3Lys_2-III (**12**) (—○—) (panel **A**) and Gal_3Lys_2-II (**8**) (—○—) (panel **B**).

arm. It was then reacted as above, after activation of its carboxylic function, with L-lysyl-L-lysine to yield, after deprotection, the triantennary ligand **12**.

Gal_3Lys_2-I (**14**) (Fig. 3) is a known compound, prepared as reported previously [17, 19]. The bi-antennary Gal_2Lys-I (**13**) (Fig. 3) was synthesized by reaction of the thio-galactose derivative **9** (Fig. 2) with lysine followed by deprotection of the hydroxyl groups.

Intergalactose distances Molecular models (Dreiding) of the synthetic tri-antennary ligands gave, when considering the S-atoms of the three thio-galactosyl moieties at the apexes of a triangle, the following distances: Gal_3Lys_2-I (**14**):1.4–1.5–2.4 nm; Gal_3Lys_2-II (**8**): 2.2–2.5–3.2 nm and Gal_3Lys_2-III (**12**): 2.5–3.0–4.3 nm. These distances, owing to the flexibility of the spacer arms, were calculated from triangles which yielded the largest sides. It appears that **14** should not meet the optimal distances of the 'golden triangle' as defined by Lee *et al.* [14]; compounds **8** and **12**, however, should be able to fit these requirements.

Table 1 Estimated dissociation constants for the binding of the synthetic multi-antennary galactosyl ligands to the Gal/GalNAc receptor of HepG2 cells and rat hepatocytes.

Ligands	K_i (M)	
	HepG2 cells	*Rat hepatocytes*
Gal_2Lys-I (**13**)	4.54×10^{-3}	-
Gal_2Lys-II (**7**)	1.73×10^{-3}	-
Gal_3Lys_2-I (**14**)	9.21×10^{-4}	4.04×10^{-5}
Gal_3Lys_2-II (**8**)	6.30×10^{-5}	5.90×10^{-6}
Gal_3Lys_2-III (**12**)	1.22×10^{-4}	5.40×10^{-6}

The K_i values were calculated from the I_{50} values, with the Cheng-Prusoff equation, using the values of K_d = 5.4 and 3.3×10^{-9} M respectively for the HepG2 and rat hepatocytes receptors.

Binding constants The potency of the synthetic bi- and tri-antennary galactosyl molecules (**7**, **8**, **12–14**) as ligands of the Gal/GalNAc receptor was examined. To that end, their ability to competitively displace ^{125}I-ASOR binding from HepG2 cells was determined. This cell line, which derives from a human hepatoblastoma, has previously been shown to express a homogeneous population of galactose receptors (their density depending on the culture conditions [28]) which present high affinity sites for ^{125}I-ASOR [18]. Under our experimental conditions, we found for this ligand a K_d of 5.4 ± 1.3 nM (not shown), a value which is in good agreement with the literature, e.g. [18]. Representative displacement experiments are shown in Fig. 4A; in all cases the fit of the curves to the data points indicated a monophasic displacement mechanism. From such curves I_{50} values were determined and corresponding K_i calculated (Table 1). It appears that, as expected from the known galactose 'clustering' effect on the affinity of the Gal/GalNAc receptor, the tri-antennary compounds were better bound than the corresponding bi-antennary ones. For example, binding of **8** is increased by a factor of 27 compared to **7**. Moreover, the inter-galactose distances also appeared to play a role. Among the tri-antennary ligands, the best ligand was **8**, which binds about 15-fold better than **14**. As discussed above, the ligand **14** has spacer-arms too short to fit the optimal triangle found by Lee *et al.* [14]. In contrast, ligand **12**, which has even greater inter-galactose distances than **8**, has a somewhat reduced affinity. This might indicate an entropic cost due to a greater flexibility.

One can therefore conclude that simplified multi-antennary galactosyl ligands, of relatively low molecular weight, can be synthesized that also comply with the known preference of the Gal/GalNAc receptor for its ligand structures. Nevertheless, the K_i values found for the tri-antennary galactosyl ligands **8, 12** and **14** (Table 1) remain relatively modest when compared with the nanomolar ranges determined for the most potent natural (or synthetic analogues) tri-antennary oligosaccharides with hepatocytes [25]; moreover it was estimated that a single galactose residue can account for up to 10^{-3} M in the overall binding process. We have therefore also investigated the affinity of our tri-antennary galactosyl ligands for the receptor of rat hepatocytes (Fig. 4B and Table 1). Compared to their binding to HepG2 cells, these ligands gained one order of magnitude in affinity, the best being **8** and **12** which now have K_i values in the micromolar range. The reasons for the differences in binding efficiency between these two galactose receptors is not clear. The hepatic Gal/GalNAc receptor has a complex hetero-oligomeric structure [29], each polypeptide chain binding a single galactose residue. Therefore, the highly precise binding of multiantennary ligands to the receptors might be sensitive to the subunit stoichiometry which are known to be different in rat hepatocytes and in HepG2 cells [29]. Nevertheless, compared to other synthetic galactose-based multi-antennary structures [14, 25 – 27], Gal_3Lys_2-II (**8**) is a promising ligand. Its relatively simple structure should make it useful for hepatocyte targeting. Thus **8**, conjugated to phosphatidylethanolamine via a spacer-arm according to the methods we have developed previously [17], gave a neo-galactolipid, which in combination with lipospermine (Transfectam®), was successfully used for targeted transfection of HepG2 cells [30]. In the future, the affinity of **8** for the Gal/GalNAc receptor also could in principle, be, further improved by e.g. replacing the galactose by GalNAc moieties. GalNAc is known to bind better to the receptor by one order of magnitude and Lee has reported that such a change in a tri-antennary structure was responsible for a 10^3-fold increased affinity [25, 26].

Conclusion

We have devised a versatile strategy that gives access to highly flexible multi-antennary galactose ligands differing in their inter-galactose distances. These synthetic compounds bind to the Gal/GalNAc receptor, expressed at the surface of HepG2 cells, in a fashion expected from studies with more complex oligomeric structures, i.e. the binding affinity order is tri- > bi-antennary ligands, and this clustering effect was also found to be sensitive to the inter-galactose distances. These ligands, that can be easily conjugated to bioactive (macro)molecule carrier systems, could be useful for targeting to the hepatocytes.

Acknowledgements

We wish to thank Mrs Liliane Italiano (Faculty of Pharmacy, Strasbourg-Illkirch) for her invaluable help in the preparation of hepatocytes.

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