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Solid-phase Synthesis of Lysine-based Cluster Galactosides with High Affinity for the Asialoglycoprotein Receptor

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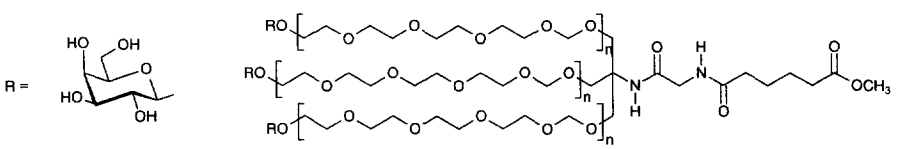
ABSTRACT

Structurally well defined di- and tri-antennary lysine-based galactose- and *N*-acetylgalactosamine-containing ligands for the hepatic asialoglycoprotein receptor (ASGP-R) could be assembled on a solid support, using a combined Fmoc/Alloc protecting group strategy for the amino functions of lysine. This methodology allowed easy introduction of spacers, the length of which could be readily accommodated for optimal binding to the ASGP-R. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

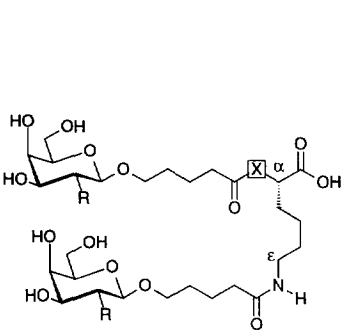
Recognition and uptake of β-D-galacto- or 2-acetamido-2-deoxy-β-D-galactopyranosyl-terminated glycoproteins by the hepatic asialoglycoprotein receptor (ASGP-R), uniquely localized on parenchymal liver cells, is a high-affinity and high-capacity process.[1,2] The latter features were an incentive to use ligands for this receptor as a targeting device for the specific delivery of drugs[3] or genes[4] to parenchymal liver cells. In order to achieve this goal, the availability of high-affinity ligands is imperative. To meet these demands, the bifunctional amino acids glutamic acid,[5] aspartic acid[6] or lysine[7] served as branching elements in the construction of multiantennary galactose-containing ligands. *In vitro* binding studies of these synthetic, as well as naturally occurring[8] cluster galactosides with the ASGP-R revealed *inter alia* that a peripheral arrangement of at least three terminal galactosides resulted in effective recognition. Recent studies from this laboratory[9]

Figure 1. Structure of TRIS-based clustergalactosides.

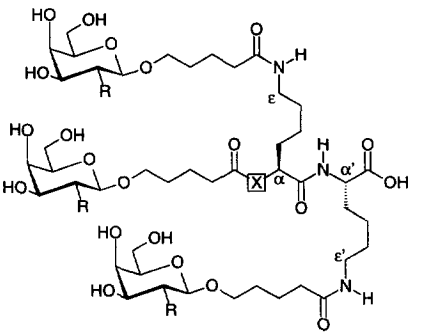


1: n = 1 (20Å)
2: n = 0 (4Å)

Figure 2. Structure of di- and triantennary galactosides **3-6** and **7-10**, respectively.



3: X = HN, R = OH
4: X = HN, R = NHAc
5: X = $HNCH_2C(O)HN$, R = NHAc
6: X = $HN(CH_2)_3C(O)HN$, R = NHAc



7: X = HN, R = OH
8: X = HN, R = NHAc
9: X = $HNCH_2C(O)HN$, R = NHAc
10: X = $HN(CH_2)_3C(O)HN$, R = NHAc

showed that a higher ligand-affinity could be attained by increasing the spacerlength in 1,1,1-tris-(hydroxymethyl)aminomethane-based (TRIS) triantennary galactosides. For example, the TRIS galactoside **1**, having a 20Å spacer, exhibited a 2000-fold higher affinity for the ASGP-R than the corresponding 4Å spacer ligand **2** (see Fig. 1).

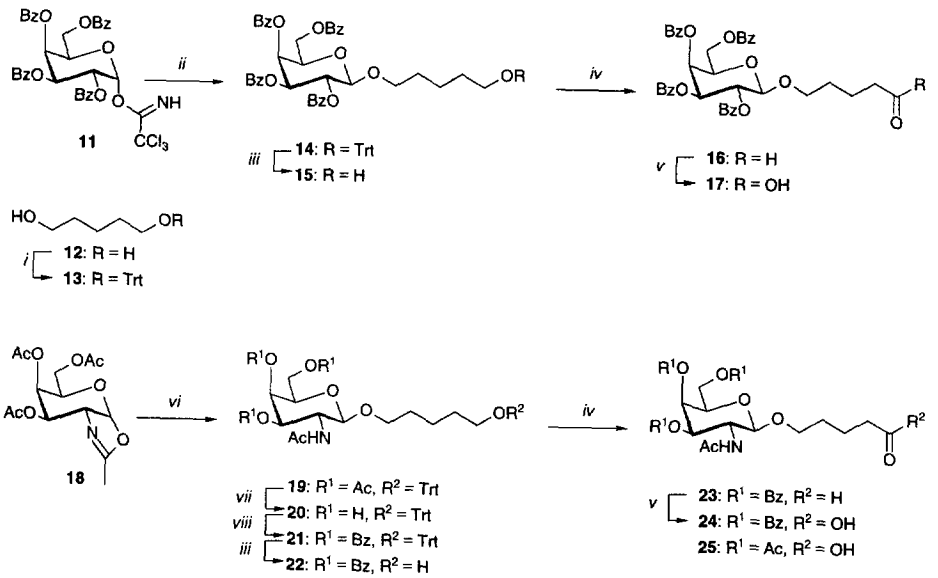
Unfortunately, further exploitation of this interesting effect was hampered by the laborious route of synthesis of this type of ligands. It occurred to us that replacement of the TRIS core-unit by lysine would allow the introduction of spacers, the length of which can be readily accommodated. This approach will be demonstrated in a solid-phase synthesis of the di- and triantennary galactosides **3-6** and **7-10**, respectively (see Fig. 2). Moreover, the influence of the spacerlength in compounds **3-10** on binding by the ASGP-R will be evaluated.

RESULTS AND DISCUSSION

The diantennary target cluster galactosides **3-6** are characterized by the presence of one lysine unit, the N^{α} and N^{ε} of which are either anchored to two galactose (as in **3**) or *N*-acetyl-galactosamine units (as in **4-6**). Furthermore, a glycine- (as in **5**) or γ-aminobutyric acid-moiety (as in **6**) is incorporated between the N^{α} of lysine and the galactosamine residue. On the other hand, in the triantennary ligands **7-10** three galactosyl units are tethered to the N^{α}, N^{ε} and $N^{\varepsilon'}$ positions of the lysyl-lysine dipeptide.

The 5-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyloxy)pentanoic acid and 5-(2-acetamido-3,4,6-tri-*O*-benzoyl-2-deoxy-galactopyranosyloxy)pentanoic acid building blocks **17** and **24**, as required for coupling with the amino functions of lysine, were prepared by the sequence of reactions outlined in Scheme 1. Condensation of known[10] 2,3,4,6-tetra-*O*-benzoyl-α-D-galactopyranosyl trichloroacetimidate (**11**) with 5-trityloxy-pentan-1-ol (**13**), readily accessible by monotritylation of pentane-1,5-diol (**12**), under the agency of a catalytic amount of $BF_3{\cdot}OEt_2$ gave the fully protected β-galactoside **14**. Acid-mediated removal of the trityl group in **14** proceeded smoothly to give the partially protected compound **15**. Swern oxidation of the primary alcohol in **15** and further elaboration[11] of aldehyde **16** with sodium chlorite in the presence of the

Scheme 1[a]

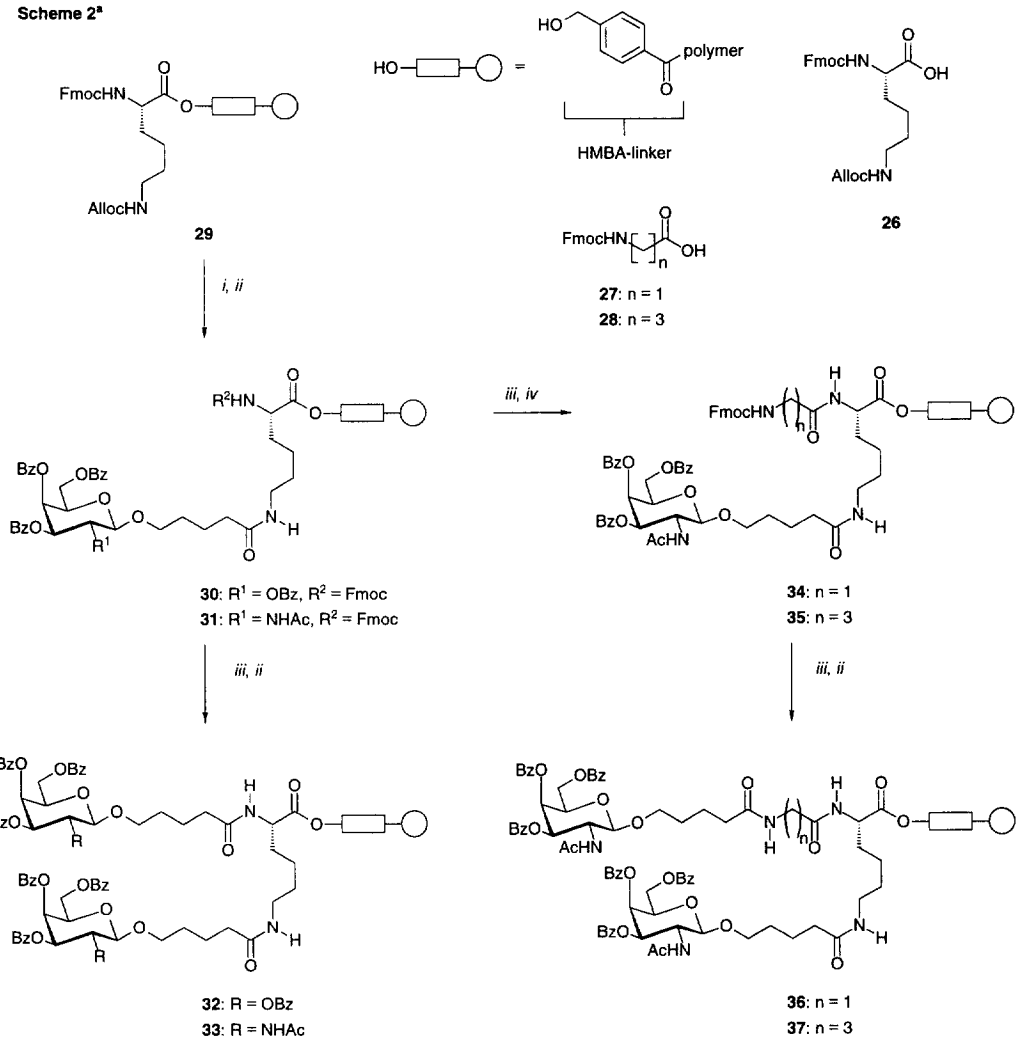


[a]Reagents and conditions
i. Trityl chloride (0.4 eq), 80%. *ii.* **13**, $BF_3{\cdot}OEt_2$, 76%. *iii.* HCOOH, MeOH, 50°C, **15**: 81%, **22**: 79%. *iv.* Oxalyl chloride, DMSO, triethylamine, 97%. *v.* $NaClO_2$, 2-methyl-2-butene, 99%. *vi.* **13**, TMSOTf (0.1 eq), 75%. *vii.* KO*t*-Bu, MeOH. *viii.* Bz_2O, 80% (two steps).

scavenger 2-methyl-2-butene led to the appropriately protected galactose derivative **17** in an overall yield of 59% based on **9**. The first step in the synthesis of the galactosamine building-unit **24** entails glycosidation of the known[12] oxazoline **18** with pentane derivative **13**. Thus, condensation of donor **18** with acceptor **13** in the presence of a catalytic amount of trimethylsilyl triflate (TMSOTf) gave compound **19**, detritylation and oxidation of which would afford the acetylated acid derivative **25**. Unfortunately, isolation of the polar detritylated product **19** (R^2=H) proved to be rather time-consuming. Alternatively, deacetylation of **19** followed by benzoylation of triol **20** gave the fully protected derivative **21**. Detritylation of **21** and subsequent two-step oxidation of the alcoholic function in **22**, under the same conditions as described for the conversion of **13** → **15**, led to the isolation of carboxylic acid derivative **24** in an overall yield of 46% over the six steps.

Having the required sugar building blocks in hand, attention was focused on the solid-phase construction of target compounds **3-10**. The fully protected and immobilized precursors **32**, **33**, **36** and **37** of the corresponding diantennary cluster galactosides **3-6** were assembled, as depicted in Scheme 2, by a combined Fmoc/Alloc solid-phase strategy using a Milligen 9050 continuous flow apparatus and commercially available polyethyleneglycol-polystyrene as the solid support. Functionalization of the resin was accomplished by BOP-assisted condensation with the commercially available base labile linker 4-(hydroxymethyl)benzoic acid (HMBA). The use of this particular linker allows cleavage from the solid-support and debenzoylation in one step. Attachment of the first amino acid Fmoc-Lys(Alloc)-OH (**26**) was effected by treating the HMBA-functionalized resin with the symmetric anhydride of **26**. Work up and further processing gave immobilized Fmoc-Lys(Alloc) (**29**) having a loading capacity of 0.18 mmol/g. Cleavage of the Alloc group

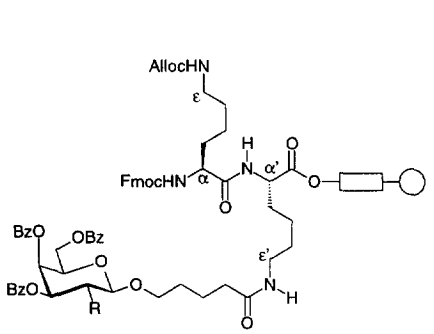
Scheme 2[a]



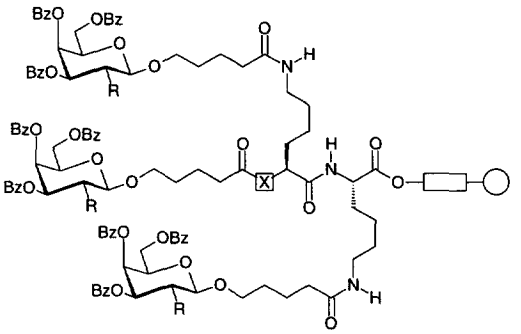
[a]**Reagents and conditions**
i. $Pd(PPh_3)_4$, HOAc/NMM/$CHCl_3$ (5/2.5/92.5, v/v/v). *ii.* **17** or **24**, BOP, DiPEA. *iii.* Piperidine/DMA (2/8, v/v). *iv.* **27** or **28**, BOP, DiPEA.

in **29** proceeded smoothly under the influence of palladium tetrakis(triphenylphosphine)[13] and was followed by BOP-mediated condensation of the free amino function with the carboxylic acid containing galactose derivative **17** to afford **30**. Removal of the Fmoc group in **30** with piperidine and subsequent elongation with the galactose derivative **17** under the influence of BOP gave the fully protected and immobilized precursors **32** of the diantennary galactoside **3**. In a similar sequence of reactions, elaboration of immobilized lysine derivative **29** with the galactosamine building block **24** led to the precursor **33** of the diantennary ligand **4**. On the other hand, extension of **31** with either Fmoc-glycine **27** (→**34**) or Fmoc-γ-aminobutyric acid **28** (→ **35**), followed by Fmoc cleavage and coupling with **24**, yielded compounds **36** and **37**, respectively.

Figure 3. Intermediates in the preparation of triantennary structures 7-10.



38: R = OBz
39: R = NHAc

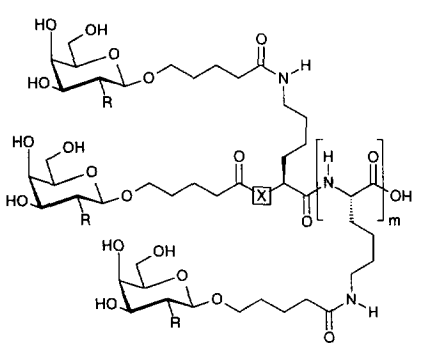


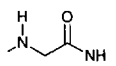
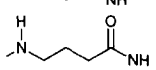
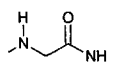
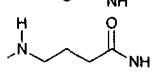
40: X = HN, R = OBz
41: X = HN, R = NHAc
42: X = $HNCH_2C(O)HN$, R = NHAc
43: X = $HN(CH_2)_3C(O)HN$, R = NHAc

The first step in the assembly of the fully protected triantennary structures **40-43** involves condensation of Fmoc-Lys(Alloc)-OH (**26**) with the free amino function of the individual immobilized galacto- and *N*-acetylgalactosamine derivatives **30** (R^2 = H) and **31** (R^2 = H) to give the corresponding immobilized Lys-Lys monosaccharide adducts **38** and **39** (see Fig. 3). The presence in **38-39** of the orthogonal Fmoc and Alloc protective groups in allows a straightforward transformation (*cf.* **29** → **32-33** and **36-37**) of **38-39** into **40-43**.

Deprotection and release from the solid support of the fully protected and immobilized galactosides **32**, **33**, **36**, **37** and **40-43** was achieved by treatment with sodium hydroxide (1 M) resulting in the crude compounds **3-10**. Purification of the individual products by gel filtration led to the isolation of the target ligands **3-10** in 50-60% yield. The identity and homogeneity of **3-10** were firmly established by ^{1}H- and ^{13}C-NMR spectroscopy as well as mass spectrometry.

Table 1



Compound	R	m	X	Ki (nM)
3	OH	0	NH	95,000
4	HNAc	0	NH	30
5	HNAc	0		470
6	HNAc	0		27
7	OH	1	NH	650
8	HNAc	1	NH	4
9	HNAc	1		10
10	HNAc	1		3

The affinity of compounds **3-10** for the ASGP-R, as monitored by an *in vitro* competition assay for ^{125}I-ASOR binding to the parenchymal liver cell, is recorded in Table 1. It can be seen that the *N*-acetylgalactosamine-containing cluster molecules are, as expected,[14] more effective ligands than the corresponding galactose-containing derivatives (*cf.* compounds **3** and **7** *vs.* **4** and **8**). It is also evident that the triantennary compounds **7-10** have a higher affinity for the ASGP-R than the corresponding diantennary compounds **3-6**.[14] Incorporation of a glycine unit at N^{α} of lysine (*i.e.* as in compound **5**) in the diantennary series led to an unexpected[9] decrease of affinity. A similar phenomenon, although less pronounced, was observed for compound **9** in the triantennary series. The latter observation may be ascribed to a restricted conformational flexibility of the glycyl-lysine moiety in cluster galactosides **5** and **9** in comparison with the parent compounds **4** and **8**. As expected, the presence of the conformationally less restricted γ-aminobutyric acid unit in the adducts **6** and **10** had only little effect on the affinity. Finally, it may be concluded that the affinity of these lysine-based *N*-acetylgalactosamine containing cluster molecules is marginally influenced by the spacerlength.

In conclusion, we have presented an efficient solid-phase preparation of a series of cluster galactosides having high affinity for the asialoglycoprotein receptor. This flexible methodology can be readily adopted for the construction and optimization of ligands for other carbohydrate-recognizing receptors.

ACKNOWLEDGMENT

These investigations are supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Technology Foundation (STW).

EXPERIMENTAL

General methods and materials

N-Methyl-2-pyrrolidone (peptide grade) was purchased from Biosolve and used without further purification. Methanol was dried by refluxing with magnesium methoxide and then distilled. Toluene, dichloromethane and 1,2-dichloroethane were distilled from P_2O_5. Pyridine and was dried by refluxing 18 h with calcium hydride and then distilled. Acetonitrile (p.a. Rathburne) was dried by storage over molecular sieves (0.4 nm). Diethyl ether was distilled from $LiAlH_4$. Methanol was stored over molecular sieves (0.3 nm) Toluene and diethyl ether were stored over sodium wire. Pyridine, dichloromethane and 1,2-dichloroethane were stored over molecular sieves (0.4 nm). Reactions were performed under anhydrous conditions at 20°C unless stated otherwise. Evaporation of solvents was performed under reduced pressure at 40°C. TLC-analyses were conducted on DC Fertigfolien (Schleicher & Schüll F 1500, LS 254). Compounds were visualised with UV light (254 nm) and by charring with concentrated sulfuric acid/ethanol (1/4, v/v). Column chromatography was performed on columns of silica gel 60, 230-400 mesh (Merck). Solid-phase synthesis was performed on a Milligen 9050 continuous flow peptide synthesizer. Tentagel S having a loading capacity of 0.26 mmol/g was purchased from RAPP Polymere. Fmoc-Lys(Alloc)-OH (**26**) was commercially available from Millipore.

^{1}H-NMR (200 MHz) and ^{13}C-NMR (50.1 MHz) spectra were recorded using a Jeol JNM-FX-200 spectrometer. Spectra were also recorded using a Bruker DMX-600 spectrometer (^{1}H-NMR: 600 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard.
Mass spectra of compounds dissolved in methanol-water (4/1, v/v) were recorded with a Finnigan MAT TSQ-70 equipped with a custom-made Electrospray Interface (ESI).

5-(Trityloxy)pentan-1-ol (13)

Pentane-1,5-diol (**12**, 10 ml, 100 mmol) was dissolved in pyridine and trityl chloride (11.2 g, 40 mmol) was added. After stirring for 16 h, the reaction was quenched with methanol and the solvents were removed by evaporation. The residue was redissolved in ether, washed with H_2O, $NaHCO_3$ (10% in H_2O), H_2O, dried ($MgSO_4$) and concentrated. The crude oil was chromatographed over silica gel (elution: light petroleum/ether 1/0 → 1/1, v/v) to give compound **13** in 80% yield (based on trityl chloride). ^{13}C{^{1}H} NMR ($CDCl_3$) δ 144.4 (C_q Trt), 128.6, 127.7, 126.8 (CH-arom), 63.4, 63.3 (CH_2OH and CH_2OTrt), 32.6, 29.8, 22.4 (CH_2).Anal. calcd. for $C_{24}H_{26}O_2$ (346.4728): C 83.2, H 7.6; found: C 83.3, H 7.5%.

5-Trityloxypentyl 2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranoside (14)

Imidate donor **11** (7.4 g, 10 mmol) and acceptor **13** (3.9 g, 11 mmol) were dissolved in 1,2-dichloroethane and crushed molecular sieves (0.4 nm, 1 g) were added. After stirring for 30 min, the reaction was started by addition of $BF_3{\cdot}OEt_2$ (0.1 ml, 1 mmol). After stirring for 3 h, TLC-analysis (light petroleum/ether 1/1, v/v) showed complete conversion of starting material and the reaction was stopped by addition of triethylamine (1 ml). The reaction mixture was filtered over a path of celite and the filtrate was diluted with dichloromethane. The mixture was washed with H_2O, $NaHCO_3$ (10%), H_2O, dried ($MgSO_4$) and concentrated. Purification of the crude mixture by silica gel column chromatography (eluent: light petroleum/ether 1/0 → 1/2, v/v) gave pure **14** (yield: 74%).
^{13}C{^{1}H} NMR ($CDCl_3$) δ 165.0-165.7 (C(O) Bz), 144.3 (C_q Trt), 129.3, 129.2, 128.9 (C_q Bz), 126.7-128.5 (CH-arom.), 101.5 (C-1), 86.1 (C_q Trt), 70.2 (C-6), 71.1, 71.1, 69.8, 68.1 (C-2, C-3, C-4, C-5), 63.1, 61.9 (CH_2O spacer), 29.4, 29.1, 22.3 (CH_2 spacer). Anal. calcd. for $C_{58}H_{52}O_{11}$ (925.0525): C 75.3, H 5.7; found: C 75.2, H 5.5%.

5-Hydroxypentyl 2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranoside (15)

Compound **14** (4.6 g, 5 mmol) was dissolved in a solution of formic acid (40 ml) in methanol (60 ml) and heated at 50°C for 6 h. The reaction mixture was slowly poured onto solid sodium carbonate and diluted with ether and water. The aqueous layer was separated and extracted three times with ether. The combined organic layers were washed with water, dried ($MgSO_4$) and concentrated. Purification of the crude oil by silica gel column chromatography (eluent: light petroleum/ethyl acetate 1/0 → 1/1, v/v) gave pure **15** in a yield of 72%.
^{13}C{^{1}H} NMR ($CDCl_3$) δ 165.0-165.5 (C(O) Bz), 128.8-129.2 (C_q Bz), 125.0-133.4 (CH-arom.), 101.5 (C-1), 70.1 (C-6), 71.5, 71.0, 69.7, 68.0 (C-2, C-3, C-4, C-5), 62.1, 61.8 (CH_2O spacer), 32.0, 28.9, 21.8 (CH_2 spacer).
Anal. calcd. for $C_{39}H_{38}O_{11}$ (682.7297): C 68.6, H 5.6; found: C 68.4, H 5.5%.

5-(2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyloxy)pentanal (16)

To a cooled (-60°C) solution of oxalyl chloride (4.7 mmol, 413 µl) in dichloromethane (6 ml) was added a solution of DMSO in dichloromethane (10 mmol, 2M, 5 ml). After stirring for 5 min a solution of alcohol **15** (2.3 mmol, 1.6 g) in dichloromethane (3 ml) was added dropwise. Stirring was continued for 30 min before triethylamine (20 mmol, 2.8 ml) was added. The reaction mixture was allowed to warm to room temperature and when TLC-analysis (light petroleum/ether 1/1, v/v) showed completion of reaction the mixture was diluted with ethyl acetate, washed with H_2O, 10% $NaHCO_3$ and H_2O, dried ($MgSO_4$) and concentrated to drieness. Purification of the crude oil by silica gel column chromatography (eluent: light petroleum/ethyl acetate 1/0 → 1/1, v/v) gave pure **16** in a yield of 97%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 201.9 (C(O) spacer), 165.4-165.8 (C(O) Bz), 128.9-129.3 (C_q Bz), 128.1-133.4 (CH-arom.), 101.5 (C-1), 69.6 (C-6), 71.5, 71.2, 69.7, 68.0 (C-2, C-3, C-4, C-5), 61.9 (CH_2O spacer), 43.0, 28.6, 18.4 (CH_2 spacer).

5-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyloxy)pentanoic acid (17)

Aldehyde **16** (0.69 mmol, 470 mg) was dissolved in a mixture of NaH_2PO_4 (4.6 mmol, 554 mg), $NaClO_2$ (80%, 4.9 mmol, 554 mg) and 2-methyl-2-butene (45 mmol, 4.75 ml) in *tert*-butanol/water (25.4 ml, 1/1, v/v). After stirring for 16 h the reaction mixture was diluted with ethyl acetate. The water layer was extracted three times with ethyl acetate and the combined organic layers were dried ($MgSO_4$) and concentrated. Purification of the crude oil by silica gel column chromatography (eluent: light petroleum/ethyl acetate 1/0 → 1/1, v/v) gave pure **17** in a yield of 99%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 177.7 (C(O) spacer), 165.0-165.8 (C(O) Bz), 128.8-129.2 (C_q Bz), 127.4-133.3 (CH-arom.), 101.4 (C-1), 69.5 (C-6), 71.5, 71.0, 69.7, 68.0 (C-2, C-3, C-4, C-5), 61.8 (CH_2O spacer), 33.0, 28.5, 20.8 (CH_2 spacer).

5-Trityloxypentyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranoside (19)

To a solution of oxazoline **18** (2 mmol, 660 mg) and 5-(trityloxy)pentan-1-ol (**13**, 3 mmol, 1.1 g) in 1,2-dichloroethane (10 ml) was added crushed molecular sieves (0.4 nm, 500 mg). After stirring for 30 min, the reaction was started by addition of TMSOTf (0.2 mmol, 40 µl). After stirring for 48 h, TLC-analysis (toluene/ethyl acetate 1/2, v/v) showed complete conversion of starting material and the reaction was stopped by addition of triethylamine (1 ml). The reaction mixture was filtered over a path of celite and the filtrate was diluted with dichloromethane. The mixture was washed with H_2O, $NaHCO_3$ (10%), H_2O, dried ($MgSO_4$) and concentrated. Purification of the crude mixture by silica gel column chromatography (eluent: toluene/ethyl acetate 1/0 → 1/2, v/v) gave pure **19** (yield: 75%).

$^{13}C\{^1H\}$ NMR δ 170.3-170.5 (C(O) Ac), 144.1 (C_q Trt), 126.7-128.5 (CH-arom.), 101.0 (C-1), 86.1 (C_q Trt), 69.6 (C-6), 70.5, 70.0, 66.9 (C-3, C-4, C-5), 62.4, 61.6 (CH_2O spacer), 51.5 (C-2), 30.1, 28.9, 22.2 (CH_2 spacer), 23.3 (CH_3 NHAc), 20.7 (CH_3 Ac).

5-Trityloxypentyl 2-acetamido-3,4,6-tri-*O*-benzoyl-2-deoxy-β-D-galactopyranoside (21)

Compound **19** (6 mmol, 4 g) was dissolved in methanol (60 ml) and treated with KO-*t*Bu (0.5 mmol, 56 mg). After stirring overnight at room temperature the reaction mixture was concentrated to dryness and traces of methanol were removed from the residue by evaporation with pyridine. The residue was redissolved in pyridine and benzoic anhydride (25 mmol, 5.6g) was added. The resulting solution was stirred for 16 h and

the reaction was stopped by addition of methanol (5 ml). After evaporation of the solvent, the remainder was taken up in ethyl acetate and washed with H_2O, 10% $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated. Purification of the crude mixture by silica gel column chromatography (eluent: toluene/ethyl acetate 1/0 → 1/2, v/v) gave pure **21** (yield: 80% over the two steps).
$^{13}C\{^1H\}$ NMR δ 170.7 (C(O) NHAc), 162.0-165.6 (C(O) Bz), 144.0 (C_q Trt), 128.8 (c_q Bz), 126.5-134.2 (CH-arom.), 100.9 (C-1), 85.9 (C_q Trt), 69.4 (C-6), 71.0, 70.7, 67.8 (C-3, C-4, C-5), 63.1, 62.8 (CH_2O spacer), 51.2 (C-2), 29.5, 29.0, 22.2 (CH_2 spacer), 22.7 (CH_3 NHAc).

5-Hydroxypentyl 2-acetamido-3,4,6-tri-*O*-benzoyl-2-deoxy-β-D-galactopyranoside (22)
Compound **22** was prepared as described above for compound **15**. Yield: 79%
$^{13}C\{^1H\}$ NMR δ 171.1 (C(O) NHAc), 165.5-165.8 (C(O) Bz), 128.8-129.1 (c_q Bz), 128.0-133.2 (CH-arom.), 101.0 (C-1), 69.4 (C-6), 70.9, 70.8, 67.8 (C-3, C-4, C-5), 62.2, 61.8 (CH_2O spacer), 51.3 (C-2), 31.7, 28.7, 21.9 (CH_2 spacer), 22.7 (CH_3 NHAc).

5-(2-Acetamido-3,4,6-tri-*O*-benzoyl-2-deoxy-β-D-galactopyranosyloxy)pentanal (23)
Compound **23** was prepared as described above for compound **16**. Yield: 97%
$^{13}C\{^1H\}$ NMR δ 202.5 (C(O) spacer), 170.5 (C(O) NHAc), 165.6-165.9 (C(O) Bz), 128.8-129.0 (c_q Bz), 128.2-133.3 (CH-arom.), 101.0 (C-1), 68.9 (C-6), 71.0, 67.9 (C-3, C-4, C-5), 62.3 (CH_2O spacer), 51.6 (C-2), 43.2, 28.6, 18.3 (CH_2 spacer), 23.0 (CH_3 NHAc).

5-(2-acetamido-3,4,6-tri-*O*-benzoyl-2-deoxy-β-D-galactopyranosyloxy)pentanoic acid (24)
Compound **24** was prepared as described above for compound **17**. Yield: 99%
$^{13}C\{^1H\}$ NMR δ 177.0 (C(O) spacer), 171.5 (C(O) NHAc), 165.5-165.9 (C(O) Bz), 128.8-129.2 (c_q Bz), 128.1-133.2 (CH-arom.), 100.7 (C-1), 68.8 (C-6), 70.9, 67.8 (C-3, C-4, C-5), 62.3 (CH_2O spacer), 51.6 (C-2), 33.2, 28.3, 21.0 (CH_2 spacer), 22.7 (CH_3 NHAc).

Solid-phase syntheses

Fmoc-Lys-(Alloc)-HMBA-Tentagel (29)
Compound **26** (2.1 g, 4.6 mmol) was dissolved in a mixture of CH_2Cl_2/dioxane (20 mL, 3/1, v/v) and DCC (0.48 g, 2.3 mmol) was added. After 10 min the resulting suspension was filtered under anhydrous conditions. The filtrate was concentrated *in vacuo*, dissolved in DMF (5 mL) and added to HMBA-Tentagel (2 g, 0.23 mmol/g) in DMF (10 mL). DMAP (56 mg, 0.46 mmol) was added and the mixture was rotated for 1 h. The reaction mixture was filtered and the residue was washed three times with DMF, *i*-PrOH and ether, respectively. The loading of the resin was estimated by suspending an aliquot of 10 mg of the resin in piperidine/DMF (0.5 mL, 1/4, v/v), diluting the suspension with MeOH to 5 mL and measuring the absorption of the fulvene adduct at 300 nm. The loading of the resin was 0.18 mmol/g.

Synthesis of the cluster galactosides 3-10
The assembly of the cluster molecules was performed on 555 mg of resin (0.1 mmol). Cleavage of the Alloc function was accomplished by treating the resin with tetrakistriphenylphosphine palladium(0) (3.1 mL, 0.07 M in acetic acid/*N*-ethylmorpholine/chloroform (5/2.5/92.5, v/v/v)) for 2 h. After Alloc cleavage, traces of metal ions were removed by washing with a solution of sodium diethyldithiocarbamate (0.5%) and

diisopropylethylamine (0.5 %) in NMP. Removal of the Fmoc group was accomplished with a solution of piperidine/NMP (1/4, v/v) according to a standard procedure for solid phase synthesis. The galactose building blocks and amino acid units were introduced according to standard protocols for solid phase synthesis. After completion of the synthesis the resin was washed successively with NMP, CH_2Cl_2 and ether. Cleavage of the cluster molecules from the solid support and saponification of the benzoyl protective groups was performed by overnight treatment with NaOH (0.5 M) at 4°C. The individual crude mixtures were purified by gel filtration (Fractogel HW 40(s) 26/60) with AcOH/water (1/99, v/v) as eluent, to give the pure compounds in 50-60% yield.

3: Yield 58%. ^{1}H NMR (D_2O) δ 4.44 (d, 2 H, 2×H1 Gal, J=8Hz), 4.31 (m, 1 H, Hα Lys), 4.09-3.51 (m, 14 H, 2×H2, H3, H4, H5, H6 Gal, C$\underline{H}_2$O spacer), 3.25 (t(b), 2 H, 2×Hε Lys), 2.46-2.27 (m, 4 H, 2×C$\underline{H}_2$C(O) spacer), 1.89-1.36 (m, 14 H, 4×C$\underline{H}_2$ spacer, 3×C$\underline{H}_2$ Lys). ^{13}C{^{1}H} NMR (D_2O) δ 177.3 (2×$\underline{C}$(O) amide), 174.5 ($\underline{C}$(O)OH), 103.4 (2×C1 Gal), 75.7, 73.5, 71.4, 69.3 (2×C2, C3, C4, C5 Gal), 70.4 (2×$\underline{C}H_2$O spacer), 61.6 (2×C6 Gal), 54.3 (Cα Lys), 39.5 (Cε Lys), 36.1, 35.6, 31.3, 28.5, 23.0, 22.6 (6×$\underline{C}H_2$ spacer, 3×$\underline{C}H_2$ Lys). Anal. Calcd for $C_{28}H_{50}N_2O_{16}$ (670.3160): C 50.1, H 7.5; found: C 50.0, H 7.5%. MS (M = 670.3) 693.1 $[M+Na]^+$.

4: Yield 55%.^{1}H NMR (D_2O) δ 4.41 (d, 2 H, 2×H1 GalNAc, J=8.5 Hz), 4.31 (m, 1 H, Hα Lys), 3.91-3.59 (m, 14 H, 2×H2, H3, H4, H5, H6 GalNAc, C$\underline{H}_2$O spacer), 3.18 (t(b), 2 H, 2×Hε Lys), 2.36-2.18 (m, 4 H, 2×C$\underline{H}_2$C(O) spacer), 2.06 (s, 6 H, 2×C$\underline{H}_3$ NHAc) 1.76-1.31 (m, 14 H, 4×C$\underline{H}_2$ spacer, 3×C$\underline{H}_2$ Lys). ^{13}C{^{1}H} NMR (D_2O) δ 177.3 (2×$\underline{C}$(O) amide), 175.1 ($\underline{C}$(O)OH), 174.8 (2×$\underline{C}$(O) NHAc), 101.9 (2×C1 GalNAc), 75.7, 71.6, 68.4 (2×C3, C4, C5 GalNAc), 70.4 (2×$\underline{C}H_2$O spacer), 61.5 (2×C6 GalNAc), 54.1 (Cα Lys), 53.0 (2×C2 GalNAc) 39.2 (Cε Lys), 36.1, 35.5, 31.5, 28.4, 23.0, 22.6 (6×$\underline{C}H_2$ spacer, 3×$\underline{C}H_2$ Lys), 22.9 ($\underline{C}H_3$ HNAc). Anal. Calcd for $C_{32}H_{56}N_4O_{16}$ (752.3690): C 51.1, H 7.5; found: C 51.3, H 7.2%. MS (M = 752.4) 751.1 $[M-H]^-$.

5: Yield 56%. ^{1}H NMR (D_2O) δ 4.41 (d, 2 H, 2×H1 GalNAc, J=8.5 Hz), 4.31 (m, 3 H, Hα Lys, 2×Hα Gly), 3.90-3.60 (m, 14 H, 2×H2, H3, H4, H5, H6 GalNAc, C$\underline{H}_2$O spacer), 3.16 (t(b), 2 H, 2×Hε Lys), 2.36-2.18 (m, 4 H, 2×C$\underline{H}_2$C(O) spacer), 2.05 (s, 6 H, 2×C$\underline{H}_3$ NHAc) 1.76-1.31 (m, 14 H, 4×C$\underline{H}_2$ spacer, 3×C$\underline{H}_2$ Lys). ^{13}C{^{1}H} NMR (D_2O) δ 177.7, 177.3 (3×$\underline{C}$(O) amide), 175.2 ($\underline{C}$(O)OH), 174.3 (2×$\underline{C}$(O) NHAc), 101.9 (2×C1 GalNAc), 75.6, 71.6, 68.4 (2×C3, C4, C5 GalNAc), 70.4 (2×$\underline{C}H_2$O spacer), 61.5 (2×C6 GalNAc), 54.1 (Cα Lys), 42.9 (Cα Gly), 53.0 (2×C2 GalNAc) 39.2 (Cε Lys), 36.1, 35.5, 31.5, 28.4, 23.0, 22.6 (6×$\underline{C}H_2$ spacer, 3×$\underline{C}H_2$ Lys), 22.6 ($\underline{C}H_3$ HNAc). Anal. Calcd for $C_{34}H_{59}N_5O_{17}$ (809.3905): C 50.4, H 7.3; found: C 50.0, H 7.2%. MS (M = 809.4) 808.5 $[M-H]^-$.

6: Yield 52%. ^{1}H NMR (D_2O) δ 4.41 (d, 2 H, 2×H1 GalNAc, J=8.5 Hz), 4.31 (m, 1 H, Hα Lys), 3.90-3.60 (m, 14 H, 2×H2, H3, H4, H5, H6 GalNAc, C$\underline{H}_2$O spacer), 3.25-3.01 (m, 4 H, 2×Hε Lys, C$\underline{H}_2$N γ-aminobutyric acid), 2.36-2.00 (m, 6 H, 2×C$\underline{H}_2$C(O) spacer, C$\underline{H}_2$C(O) γ-aminobutyric acid), 2.09 (s, 6 H, 2×C$\underline{H}_3$ NHAc) 1.89-1.31 (m, 16 H, 4×C$\underline{H}_2$ spacer, 3×C$\underline{H}_2$ Lys, C$\underline{H}_2$ γ-aminobutyric acid). ^{13}C{^{1}H} NMR (D_2O) δ 177.7, 177.3 (4×$\underline{C}$(O) amide), 174.9 ($\underline{C}$(O)OH), 174.5 (2×$\underline{C}$(O) NHAc), 102.1 (2×C1 GalNAc), 75.6, 71.6, 68.4 (2×C3, C4, C5 GalNAc), 70.3 (2×$\underline{C}H_2$O spacer), 61.5 (2×C6 GalNAc), 54.9 (Cα Lys), 53.0 (2×C2 GalNAc) 39.6, 39.2 (Cε Lys, $\underline{C}H_2$N γ-aminobutyric acid), 36.0, 33.4, 31.5, 28.7, 25.2, 23.2, 22.8 (6×$\underline{C}H_2$ spacer, 3×$\underline{C}H_2$ Lys, 2×$\underline{C}H_2$ γ-aminobutyric acid), 22.9 ($\underline{C}H_3$ HNAc). Anal. Calcd for $C_{36}H_{63}N_5O_{17}$ (837.4218): C 51.6, H 7.6; found: C 51.7, H 7.7%. MS (M = 837.4) 836.5 $[M-H]^-$.

7: Yield 55%. ^{1}H NMR (D_2O) δ 4.44 (d, 3 H, 3×H1 Gal, J=8Hz), 4.31 (m, 2 H, 2×Hα Lys), 4.09-3.51 (m, 22 H, 3×H2, H3, H4, H5, H6 Gal, C$\underline{H}_2$O spacer), 3.25 (m, 4 H, 4×Hε Lys), 2.46-2.27 (m, 6 H, 2×C$\underline{H}_2$C(O)

spacer), 1.89-1.36 (m, 24 H, 6×C$\underline{H}_2$ spacer, 6×C$\underline{H}_2$ Lys). $^{13}C\{^1H\}$ NMR (D_2O) δ 177.3, 177.1 (3×$\underline{C}$(O) amide), 174.8 ($\underline{C}$(O)OH), 103.4 (3×C1 Gal), 75.7, 73.4, 71.4, 69.3 (3×C2, C3, C4, C5 Gal), 70.4 (3×$\underline{C}H_2O$ spacer), 61.6 (3×C6 Gal), 54.3, 53.5 (2×Cα Lys), 39.5 (2×Cε Lys), 36.1, 35.6, 31.3, 30.8, 28.8, 28.5, 28.4, 23.0, 22.6 (8×$\underline{C}H_2$ spacer, 6×$\underline{C}H_2$ Lys). Anal. Calcd for $C_{45}H_{80}N_4O_{24}$ (1060.5161): C 51.0, H 7.6; found: C 50.6, H 7.5%. MS (M = 1060.5) 1083.1 $[M+Na]^+$.

8: Yield 49%. ^1H NMR (D_2O) δ 4.44, 4.43 (2d, 3 H, 3×H1 GalNAc, J=8.5 Hz), 4.31 (m, 2 H, 2×Hα Lys), 3.93-3.56 (m, 22 H, 3×H2, H3, H4, H5, H6 GalNAc, C$\underline{H}_2$O spacer), 3.18-3.14 (m, 4 H, 4×Hε Lys), 2.31-2.22 (m, 6 H, 3×C$\underline{H}_2$C(O) spacer), 2.02 (s, 9 H, 3×C$\underline{H}_3$ NHAc) 1.82-1.34 (m, 24 H, 6×C$\underline{H}_2$ spacer, 6×C$\underline{H}_2$ Lys). $^{13}C\{^1H\}$ NMR (D_2O) δ 177.3, 177.1 (3×$\underline{C}$(O) amide), 176.3 ($\underline{C}$(O)OH), 174.8 (3×$\underline{C}$(O) NHAc), 102.0 (3×C1 GalNAc), 75.6, 71.6, 68.3 (3×C3, C4, C5 GalNAc), 70.3 (3×$\underline{C}H_2O$ spacer), 61.5 (3×C6 GalNAc), 54.2, 53.0 (2×Cα Lys), 52.9 (3×C2 GalNAc) 39.7 (2×Cε Lys), 35.9, 35.5, 31.9, 31.0, 28.6, 28.4, 23.1, 23.0, 22.6, 22.4 (9×$\underline{C}H_2$ spacer, 6×$\underline{C}H_2$ Lys), 22.8 ($\underline{C}H_3$ HNAc). Anal. Calcd for $C_{51}H_{89}N_7O_{24}$ (1183.5958): C 51.7, H 7.6; found: C 51.5, H 7.2%. MS (M = 1183.6) 1182.7 $[M-H]^-$.

9: Yield 54%. ^1H NMR (D_2O) δ 4.46, 4.41 (2d, 3 H, 3×H1 GalNAc, J=8.5 Hz), 4.38 (m, 4 H, 2×Hα Lys, 2×Hα Gly), 3.91-3.51 (m, 22 H, 3×H2, H3, H4, H5, H6 GalNAc, C$\underline{H}_2$O spacer), 3.16-3.16 (m, 4 H, 4×Hε Lys), 2.30-2.22 (m, 6 H, 3×C$\underline{H}_2$C(O) spacer), 2.00 (s, 9 H, 3×C$\underline{H}_3$ NHAc) 1.82-1.34 (m, 24 H, 6×C$\underline{H}_2$ spacer, 6×C$\underline{H}_2$ Lys). $^{13}C\{^1H\}$ NMR (D_2O) δ 177.3, 177.1 (3×$\underline{C}$(O) amide), 176.2 ($\underline{C}$(O)OH), 174.9 (3×$\underline{C}$(O) NHAc), 101.8 (3×C1 GalNAc), 75.6, 71.6, 68.1 (3×C3, C4, C5 GalNAc), 70.2 (3×$\underline{C}H_2O$ spacer), 61.6 (3×C6 GalNAc), 54.2, 53.0 (2×Cα Lys), 52.9 (3×C2 GalNAc) 39.6 (2×Cε Lys), 42.8 (Cα Gly), 35.9, 35.6, 31.3, 31.1, 28.6, 28.4, 22.9, 22.7, 22.6, 22.4 (9×$\underline{C}H_2$ spacer, 6×$\underline{C}H_2$ Lys), 22.2 ($\underline{C}H_3$ HNAc). Anal. Calcd for $C_{53}H_{92}N_8O_{25}$ (1240.6172): C 51.3, H 7.5; found: C 51.5, H 7.4%. MS (M = 1240.6) 1239.4 $[M-H]^-$.

10: Yield 51%. ^1H NMR (D_2O) δ 4.41 (d, 3 H, 3×H1 GalNAc, J=8.5 Hz), 4.31 (m, 2 H, 2×Hα Lys), 3.93-3.56 (m, 22 H, 3×H2, H3, H4, H5, H6 GalNAc, C$\underline{H}_2$O spacer), 3.25-3.01 (m, 6 H, 4×Hε Lys, C$\underline{H}_2$N γ-aminobutyric acid), 2.36-2.00 (m, 8 H, 3×C$\underline{H}_2$C(O) spacer, C$\underline{H}_2$C(O) γ-aminobutyric acid), 2.12 (s, 9 H, 2×C$\underline{H}_3$ NHAc) 1.89-1.31 (m, 26 H, 6×C$\underline{H}_2$ spacer, 6×C$\underline{H}_2$ Lys, C$\underline{H}_2$ γ-aminobutyric acid). $^{13}C\{^1H\}$ NMR (D_2O) δ 177.0, 176.9, 176.4 (5×$\underline{C}$(O) amide), 175.1 (3×$\underline{C}$(O) NHAc), 174.5 ($\underline{C}$(O)OH), 102.1 (3×C1 GalNAc), 75.6, 71.6, 68.4 (3×C3, C4, C5 GalNAc), 70.4 (3×$\underline{C}H_2O$ spacer), 61.5 (2×C6 GalNAc), 54.3, 53.9 (2×Cα Lys), 52.9 (3×C2 GalNAc) 39.6, 39.1 (Cε Lys, $\underline{C}H_2N$ γ-aminobutyric acid), 35.9, 33.2, 31.2, 31.0, 28.6, 28.5, 28.4, 25.5, 23.0, 22.6 (9×$\underline{C}H_2$ spacer, 6×$\underline{C}H_2$ Lys, 2×$\underline{C}H_2$ γ-aminobutyric acid), 22.8 ($\underline{C}H_3$ HNAc). Anal. Calcd for $C_{55}H_{96}N_8O_{25}$ (1268.6485): C 52.0, H 7.6; found: C 52.3, H 7.8%. MS (M = 1268.6) 1267.6 $[M-H]^-$.

Isolation of parenchymal liver cells.

Male Wistar rats of approximately 250 g were anaesthetized by intraperitoneal injection of 20 mg of sodium pentobarbital. Parenchymal liver cells were isolated after a 20 min perfusion of the liver with collagenase (type IV, 0.05%) at 37 °C, according to the method of Seglen[15], modified as previously described[16]. Following perfusion, parenchymal cells were purified by differential centrifugation as described in detail elsewhere[17].

Iodination of asialoorosomucoïd.

Human orosomucoid was isolated and subsequently desialylated enzymatically as described[18]. The protein was radiolabeled with carrier free $Na^{125}I$ by the ICl method of McFarlane as modified by Bilheimer *et al.*.[19]

In vitro binding studies.

Displacement of ^{125}I-ASOR binding to hepatocytes was determined as follows. Parenchymal liver cells (1-1.5 10^6 cells; viability >90%) were incubated in 1 ml of Dulbecco's modified essential medium containing 2% BSA, with ^{125}I-ASOR (5.5 nM) in the presence or absence of unlabelled displacer at 8 concentrations, ranging from 1nM to 1 mM. Following incubation for 2h at 4°C under gentle agitation, the medium was removed by aspiration and the cells were washed twice with 2 ml of ice-cold medium containing 0.2% BSA and once with medium lacking BSA. Subsequently, cells were counted for radioactivity. Cell binding was corrected for protein content. Non-specific binding was measured in the presence of 100 mM GalNAc. Displacement binding data were analyzed according to a single site model using a computerized nonlinear fitting program (GraphPad)[20] to calculate the K_i.

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