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Trivalent, Gal/GalNAc-containing ligands designed for the asialoglycoprotein receptor

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Abstract—A series of novel, fluorescent ligands designed to bind with high affinity and specificity to the asialoglycoprotein receptor (ASGP-R) has been synthesized and tested on human liver cells. The compounds bear three non-reducing, β-linked Gal or Gal*N*Ac moieties linked to flexible spacers for an optimal spatial interaction with the binding site of the ASGP-R. The final constructs were selectively endocytosed by HepG2 cells derived from parenchymal liver cells—the major human liver cell type—in a process that was visualized with the aid of fluorescence microscopy. Furthermore, the internalization was analyzed with flow cytometry, which showed the process to be receptor-mediated and selective. The compounds described in this work could serve as valuable tools for studying hepatic endocytosis, and are suited as carriers for site-specific drug delivery to the liver.

1. Introduction

The asialoglycoprotein receptor (ASGP-R) is located on hepatocytes and is a Ca^{2+}-dependent carbohydrate-binding protein, or C-type lectin. It is expressed on mammalian liver cells.[1] Its main function is to maintain serum glycoprotein homeostasis by the recognition, binding, and endocytosis of asialoglycoproteins (ASGPs), that is, desialylated glycoproteins with terminal galactose or Gal*N*Ac residues. After internalization via clathrin-coated pits and their fusion with endosomes, the ASGPs are released in the acidic environment of the endosome and transported to lysosomes for degradation, while the receptor is recycled back to the cell surface.[2,3]

In addition to the ASGP-R, there are three additional Gal/Gal*N*Ac-receptors in the C-type lectin family: the Kupffer cell receptor, the macrophage galactose lectin, and the scavenger receptor C-type lectin (SRCL).[4–7] Their binding properties were recently profiled by Drickamer et al.[8]

The affinity and specificity of the ASGP-R is a consequence of oligovalent interactions with its physiological ligands, a process termed *cluster glycoside effect* by Lee et al.[9] The receptor consists of two homologous subunits, designated H1 and H2 in the human system, which form a non-covalent heterooligomeric complex with an estimated ratio of 2–5:1, respectively. Both subunits are single-spanning membrane proteins with a calcium-dependent galactose/*N*-acetylgalactosamine recognition domain.[10] Recently, the X-ray crystal structure of the carbohydrate recognition domain (CRD) of the major subunit H1 was elucidated.[11]

Many studies have been performed with both natural and synthetic carbohydrates to establish the structure–affinity relationship for the ASGP-R. Baenzinger et al.[12,13] have shown that the human receptor exhibits specificity for terminal Gal and Gal*N*Ac (with an approximately 50-fold higher affinity for the latter) on desialylated glycoproteins. Triantennary ligands displayed a higher affinity than their mono- and diantennary counterparts. Furthermore, the studies led to the conclusion that only the terminal residues are necessary for specific recognition, and that the binding process proceeds through a simultaneous interaction of 2–3 su-

Abbreviations: NIS, *N*-iodosuccinimide; TfOH, trifluoromethanesulfonic acid; NHS, *N*-hydroxysuccinimide; PyBOP, benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EEDQ, ethyl 1,2-dihydro-2-ethoxy-1-quinolinecarboxylate; DMTST, dimethyl(methylthio)sulfonium trifluoromethanesulfonate; DIPEA, diisopropylethylamine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid.

Keywords: Asialoglycoprotein receptor (ASGP-R); Drug delivery; Flow cytometry; Fluorescence microscopy; Fluorescent probes.

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gar residues with 2–3 binding sites of the heterooligomeric receptor. On the native receptor on the hepatocyte surface these binding sites are 25–30 Å apart.

Studies on rabbit hepatocytes by Lee et al.,[9,14] using synthetic oligosaccharides, further reinforced the binding hierarchy of polyvalent ligands: tetraantennary > triantennary ≫ diantennary ≫ monoantennary. The IC_{50}-values for mono-, di-, tri-, and tetraantennary oligosaccharides were found to be approximately 1×10^{-3}, 1×10^{-6}, 5×10^{-9}, and 10^{-9} M, respectively. In other words, although the number of Gal residues/mol of ligand increased only 4-fold, the inhibitory potency increased 1,000,000-fold. Because the fourth Gal moiety present in the tetraantennary ligand does not markedly enhance the affinity, it was assumed that the binding requirements of the cell-surface receptor are largely satisfied by the triantennary structure.[15]

The optimal distance of the Gal moieties in these oligosaccharides was determined by binding assays with synthetic carbohydrates representing partial structures of*N*-linked glycans,[16] high-resolution NMR, and molecular modeling studies.[17] Based on these results, Lee et al.[9,16] presented a model for the optimal spatial arrangement of the terminal sugar residues (Fig. 1).

Due to its specificity, predominant expression on hepatocytes, and high capacity for receptor-mediated endocytosis, the ASGP-R has been validated as a potential target for drug and gene delivery to the liver.[7,18,19] As an alternative to ex vivo gene transfer to the liver, which requires invasive surgery,[20] there is much interest in in vivo protocols: (i) Wu et al.[21] demonstrated successful in vivo gene transfer to hepatocytes with poly-L-lysine linked asialoorosomucoid, (ii) Hara et al.[22–24] showed that asialofetuin-labeled liposomes that encapsulate plasmid DNA cause gene expression, and (iii) successful gene transfer to hepatocytes using liposomal gene carriers that possess synthetic galactose residues as a targetable ligand for parenchymal liver cells has been reported.[25]

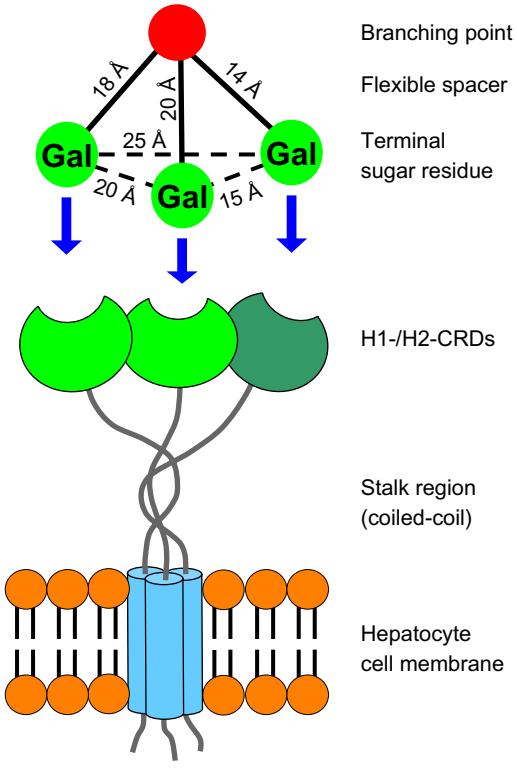


Figure 1. Binding model for ASGP-R ligands in an optimal conformation to the heterooligomeric receptor consisting of H1 and H2 subunits. Dashed line indicates the distance between the C-4 of each Gal moieties; filled line represents approximate distance between branching point and C-6 of Gal (14–20 Å). Adapted from Lee et al.[16]

In order to further exploit the ASGP-R for therapeutic purposes, trivalent ligands with pendant Gal or Gal*N*Ac residues connected by flexible spacers with appropriate lengths to a common branching point were synthesized. All these ligands incorporate 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) as the branching point (Fig. 2). Kempen et al.[26] synthesized the trivalent, Gal-terminated ligand **1**, where the carbohydrate moieties were directly linked to Tris. When **1** was labeled with cholesterol and incorporated into liposomes, they were mainly taken up by the Kupffer cells, via the Gal/Fuc-recognizing receptor, and not by the parenchymal liver cells via the ASGP-R.

Therefore, a new generation of ligands with optimal spacers was created. Biessen et al.[27,28] extended the distance between the Tris branching point and the Gal residues by using tetraethylene glycol spacers approximately 20 Å in length. This indeed led to ligands with improved affinities (see **2**, $K_i = 0.2$ μM, Fig. 2) determined in a competition assay with ^{125}I-labeled asialoorosomucoid. In 1999, Sliedregt et al.[29] designed a second generation of cluster glycosides containing an essential modification (see **3**, $K_i = 93$ nM, Fig. 2). To enhance the chemical stability, the methylene acetal groups in **2**, which connect the spacers to Tris, were replaced by acid stable ether bonds. Furthermore, the spacers were no longer based on tetraethylene glycol to achieve the appropriate spacing between the Gal residues, but rather on a twelve atom fragment containing two amide bonds. Finally, Rensen et al.[30] combined the various features from **2** and **3** to generate compound **4** ($K_i = 2$ nM, Fig. 2), which exploited the expected 50-fold higher affinity of Gal*N*Ac over Gal toward the ASGP-R.[31]

To further improve the therapeutic profile of the previously reported ligands of the ASGP-R,[26–31] we set out to synthesize the optimal trivalent linker (**19**, Scheme 1) with reduced synthetic complexity, high in vivo stability, and improved spacer flexibility. The resultant intermediates **5a** and **5b** (Fig. 2), which possess terminal Gal or Gal*N*Ac moieties, respectively, were then fluorescent-labeled and tested for selective uptake by hepatocytes using fluorescence microscopy and flow cytometry. Moreover, since most of the previous research was done on rat[26–30] and mouse[32] liver cells, and the final aim of this research is liver-selective drug delivery in humans, all our biological assays were performed using cell lines of human origin.

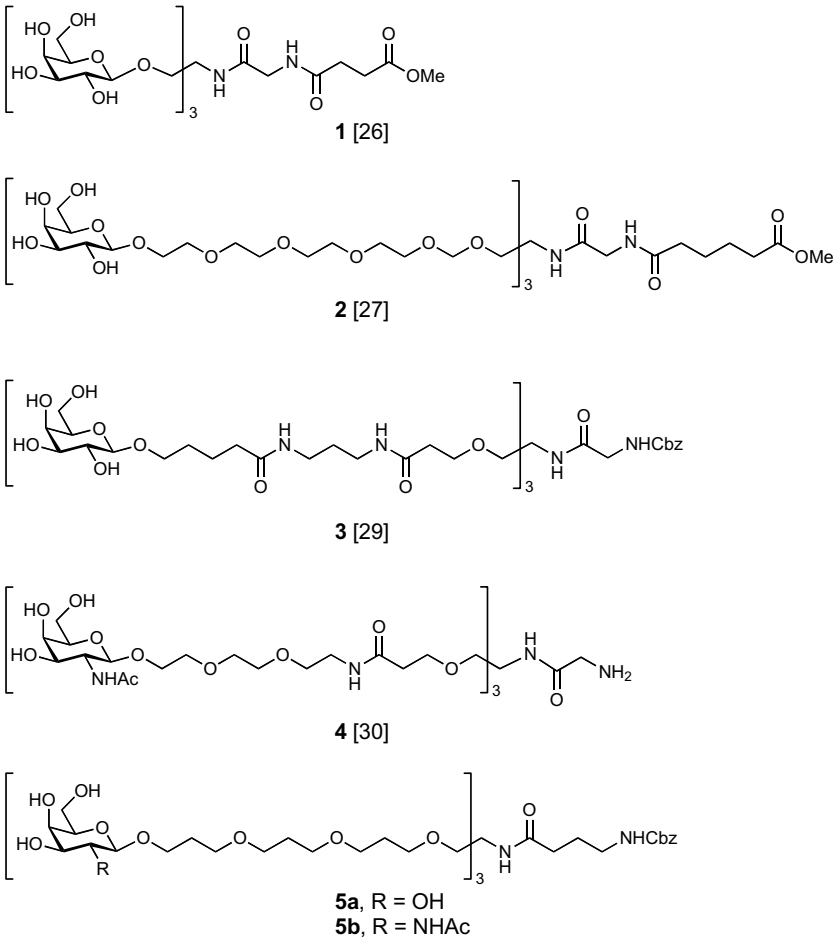


Figure 2. Trivalent compounds **1**–**4** were specifically designed for, and tested on, the ASGP-R.[26–30] Compounds **5a** and **5b** are the trivalent, Cbz-protected intermediates introduced herein.

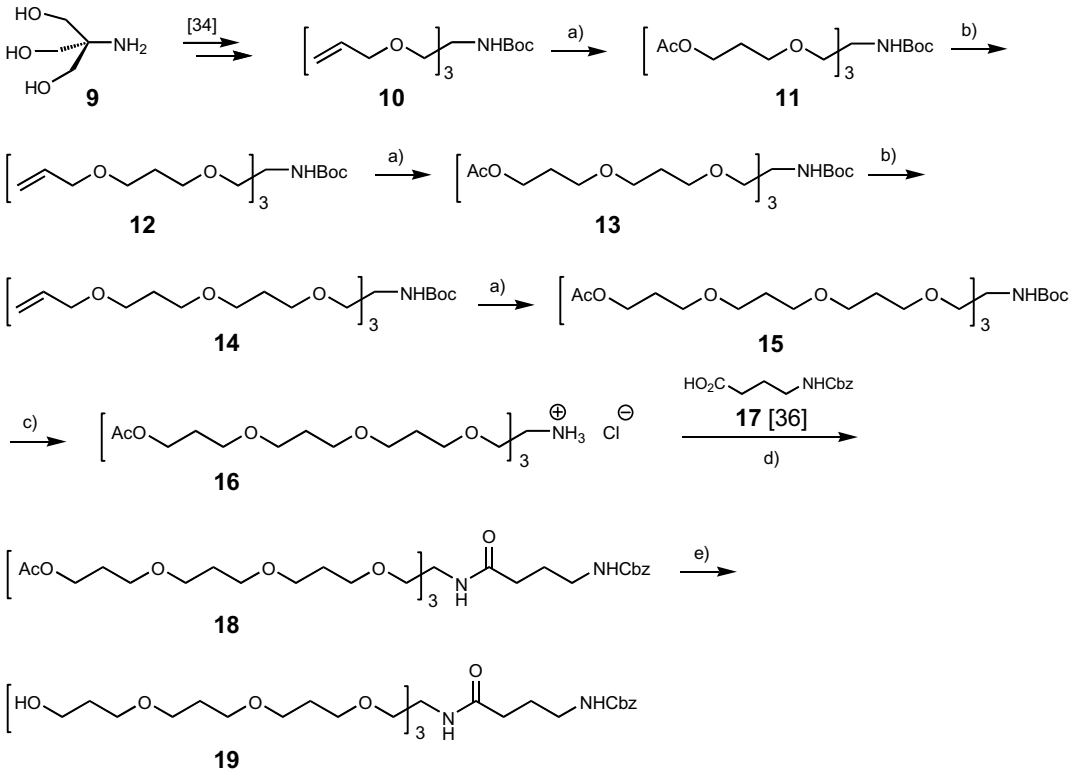
2. Results and discussion

The main structural features of the trivalent ASGP-R ligands **5a** and **5b** are as follows: (i) Tris is the central branching point, (ii) the spacers are based on polypropylene oxide, which combines flexibility with amphilicity, (iii) the linkage between Tris and the spacers is a hydrolytically stable ether bond, and (iv) the length of the spacers can be easily varied.

The glycine acylating the amino group of Tris in **4** (Fig. 2) has been replaced with Cbz-protected γ-aminobutyric acid, which upon deprotection furnishes a versatile primary amino group for the attachment of fluorescent labels and at a later stage therapeutic agents. For our studies, the amino group was coupled to Alexa Fluor® 488 fluorescent label[33] (→**6** and **7**, Fig. 3), but in theory it could also be coupled to a therapeutic agent. As a negative control for the fluorescence microscopy studies, and especially to demonstrate the significance of the polypropylene oxide spacers featured in our final compounds **6** and **7**, we also synthesized compound **8** (Fig. 3) The latter, in contrast to **6** and **7**, has only short spacers, and therefore does not fulfill the spatial requirements for trivalent binding to the ASGP-R.

2.1. Synthesis of fluorescent-labeled, trivalent ligands 6 and 7 and the negative control 8

Starting from 2-amino-2-hydroxymethyl-1,3-propanediol (Tris, **9**), the polypropylene oxide spacers were gradually extended by repetitive allylation/oxidative hydroboration steps using 9-BBN in THF followed by H_2O_2 and aqueous NaOH (Scheme 1). For the synthesis of compounds **12** and **14**, several allylation procedures were examined using NaH, KOH, K_2CO_3 as bases in various solvents (e.g., THF, DMF, dioxane), with and without the addition of crown ethers, and quaternary ammonium salts as phase transfer catalysts. All procedures, including the literature procedure used to obtain **10**[34] in 68%, led to unacceptably low yields of approximately 40% for **12**, along with a considerable amount of a tetraallylated side product. The desired triallylated compounds could finally be obtained in almost quantitative yields with only traces of *N*-allylation, by employing liquid–liquid phase transfer catalysis.[35] Thus, **12** and **14** were obtained in 95% and 90%, respectively, from the corresponding triols using allyl bromide in refluxing DCM/50% aqueous NaOH (1:1) with a catalytic amount of 15-crown-5. Oxidative hydroboration and acetylation gave **13** and **15** in excellent overall yields. The peracetylation step (→**11**, **13**, and **15**) was applied in order to



Scheme 1. Reagents and conditions: (a) i—9-BBN, THF, rt, 24 h, then aq NaOH, H_2O_2, 0 °C → rt, 24 h; ii—Ac_2O, pyridine, rt, 3 h, (**11**: 81%; **13**: 87%; **15**: 88%); (b) i—NaOMe, MeOH, rt, 24 h, quant.; ii—allyl bromide, 15-crown-5, DCM/50% (w/v) aqueous NaOH, reflux, 24 h, (**12**: 95%; **14**: 90%); (c) 4M HCl in dioxane, rt, 30 min, quant.; (d) PyBOP, DIPEA, dioxane/DMF, rt, 24 h, 85%; (e) NaOMe, MeOH, rt, 4 h, 90%.

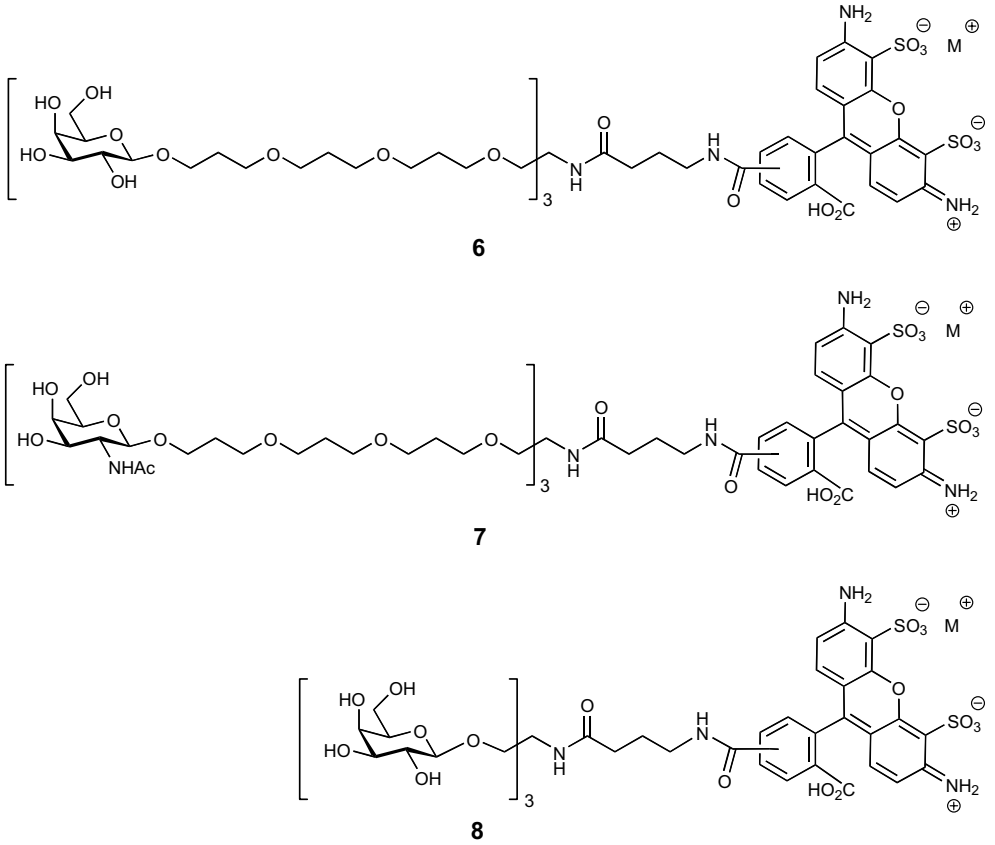


Figure 3. Fluorescent-labeled, trivalent compounds **6**, **7**, and control **8**; M^+ are variable counterions.

facilitate purification and characterization of the intermediate triols. The subsequent deacetylation of **11** and **13** was achieved under standard Zemplén conditions. For the elaboration at the *N*-terminus of **15**, the Boc protecting group was selectively removed using 4 M HCl in dioxane leading quantitatively to compound **16**. Subsequent condensation with the *N*-Cbz-protected γ-aminobutyric acid linker **17**[36] using PyBOP in DMF/dioxane (1:3) and DIPEA as base yielded **18**. In the final step, deacetylation under Zemplén conditions furnished the trivalent glycosyl acceptor **19** in an overall yield of 27%, starting from Tris (**9**).

Galactosylation of **19** with ethyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-galactopyranoside (**20**)[27] using DMTST as a promoter furnished the trivalent intermediate **21** in a 68% yield (Scheme 2). Debenzoylation (→**5a**) followed by the cleavage of the Cbz protecting group gave **22**, which was coupled to the *N*-hydroxysuccinimidyl (NHS)-activated Alexa Fluor® 488 fluorescent label to yield compound **6** in 81% yield. Alexa Fluor® 488 was found to be the optimal fluorescent label for our purposes, combining high chemical and photostability with high fluorescence intensity. An analogous sequence of reactions was applied for the synthesis of **7**. First, the *N*-acetylgalactosamine trimer **24** (Scheme 2) was obtained in 91% by glycosylating **19** with ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-galactopyranoside (**23**).[37] After the cleavage of the Troc protecting group, the free amine was directly acetylated to furnish **25**. Upon deprotection of the *N*-acetylgalactosamine moieties (→**5b**), the Cbz group was cleaved yielding compound **26**, which was labeled with Alexa Fluor® 488 producing **7** in a 90% yield.

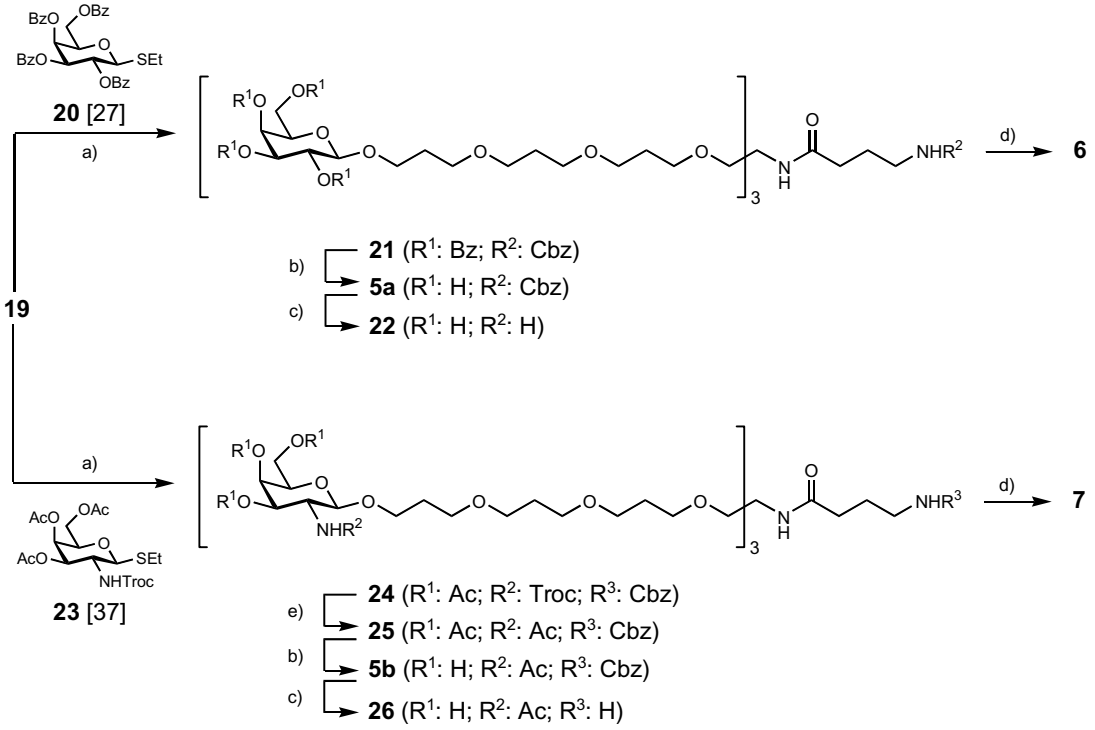
As a negative control for cellular assays, compound **8** (Scheme 3) was synthesized via the acylation of Tris (**9**) with *N*-Cbz-protected γ-aminobutyric acid (**17**)[36] using EEDQ in pyridine,[38] yielding compound **27** in a 77% yield. The latter was then galactosylated with donor **20**[27] using NIS/TfOH as a promoter to give **28** in 51%. After debenzoylation (→**29**), the Cbz group was cleaved by hydrogenolysis to furnish compound **30**, which was subsequently coupled to the *N*-hydroxysuccinimidyl (NHS)-activated Alexa Fluor® 488 fluorescent label yielding **8** in a 96% yield.

3. Biological evaluation

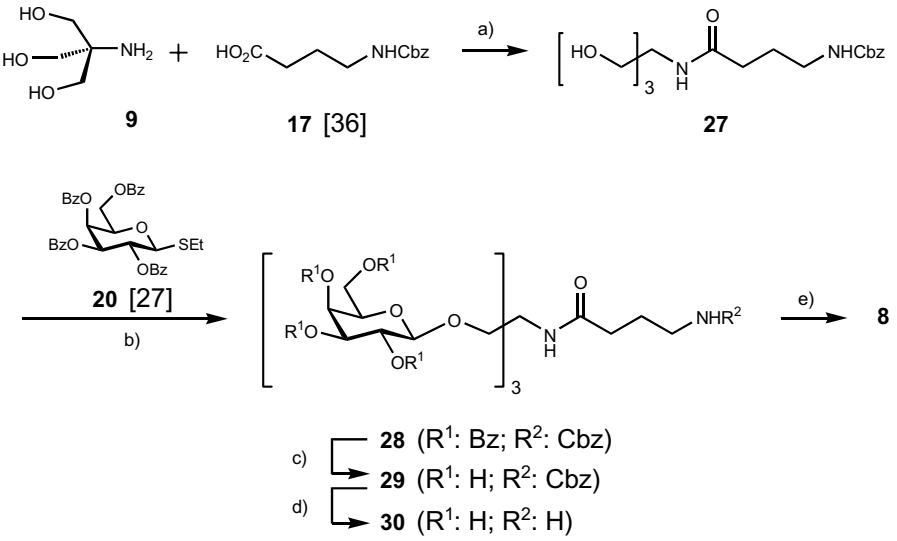
The trivalent ligands **6**–**8** were examined for their selective binding to, and internalization by the ASGP-R applying fluorescence microscopy and flow cytometry. Two different cell lines of hepatic origin were used: HepG2 cells derived from a human hepatocellular carcinoma expressing the ASGP-R,[39] and the human more endothelial-like SK-Hep1 cells, which lack the receptor.[40]

3.1. Fluorescence microscopy

The cells were incubated with the Alexa Fluor® 488-labeled compounds **6**, **7**, or **8** for 1.5 h on ice to allow binding of the compounds to the receptor while prevent-



Scheme 2. Reagents and conditions: (a) DMTST, 4 Å MS, DCM, 0 °C → 10 °C, 48–72 h, 68% for **21**, 91% for **24**; (b) NaOMe, MeOH/dioxane, rt, 4 h, 94% for **5a**, 72% for **5b**; (c) H_2, Pd/C, EtOH/dioxane, rt, 24 h, 87% for **22**, 95% for **26**; (d) Alexa Fluor® 488-NHS, DIPEA, 4 Å MS, DMF/dioxane, rt, 4 days, 81% for **6**, 90% for **7**; (e) Zn dust, Ac_2O, dioxane, rt, 24 h, 82%.



Scheme 3. Reagents and condition: (a) EEDQ, pyridine, 90 °C, 24 h, 77%; (b) NIS, TfOH, 4 Å MS, DCE/Et_2O, 0 °C, 1 h, 51%; (c) NaOMe, dioxane/MeOH, rt, 6 h, 85%; (d) H_2, Pd/C, MeOH, rt, 48 h, 87%; (e) Alexa Fluor® 488-NHS, DIPEA, 4 Å MS, DMF, rt, 4 days, 96%.

ing unspecific uptake. In the washing step, unbound ligand was removed, and the cells were incubated for an additional 40 min at 37 °C to allow receptor-mediated endocytosis of bound compounds to take place. The specific uptake led to punctuate staining of the cells representing endosomes containing the ligands, which were visualized by fluorescence microscopy. HepG2 cells showed specific uptake of **6** and **7**, and only negligible uptake of **8**. The fluorescent content of the endosomes can be distinctly seen (Fig. 4A and C) for compounds **6** and **7**, respectively. Because the cells were grown and incubated on glass coverslips, which were then mounted upside down for visualization, enriched fluorescence can only be observed in cytosolic areas, which are not blocked by the nuclei. Panel E shows little or no such fluorescent vesicles, since control compound **8** was not internalized via the ASGP-R owing to insufficient spacer length. As expected, no internalization into SK-Hep1 cells (which do not express the ASGP-R) could be observed for compounds **6** and **7** (Fig. 4B and D). However, compound **8** showed a minor tendency to be internalized by this cell line in an ASGP-R-independent manner (Fig. 4F). Panels G and H show the autofluorescence of non-treated HepG2 and SK-Hep1 cells as controls.

3.2. Flow cytometry

The ASGP-R-mediated uptake of compounds **7** and **8** (negative control) was quantitatively evaluated by flow cytometry (Figs. 5 and 6). Instead of performing the previously described steps (prebinding on ice, removal of the excess, and internalization of bound compound), the cells were continuously incubated with the test compounds at 37 °C and analyzed.

The median fluorescence intensity (MFI) of cells incubated with compound **7** at concentrations ranging from 0.4 to 12.5 μM revealed low uptake of the compound into SK-Hep1 cells compared to that of HepG2 cells, in which the uptake leads to a saturation hyperbola as it is typical for a receptor-mediated process (Fig. 5).[41]

Uptake of compound **7** into HepG2 cells via the ASGP-R at a concentration of 10 μM was competitively inhibited by the presence of monosaccharide ligands: Gal*N*Ac (IC_{50} = 4.55 ± 0.32 mM) (Fig. 6A) and asialofetuin (IC_{50} = 45.60 ± 2.70 μM) (Fig. 6B), whereas the uptake into SK-Hep1 was low and not affected by the presence of asialofetuin.

In ASGP-R bearing HepG2 cells, uptake of control compound **8** was low and proved to be unspecific as it could not be inhibited by asialofetuin, a natural high-affinity ligand of the receptor (Fig. 6C). ASGP-R-negative SK-Hep1 cells, on the other hand, evinced high uptake of compound **8**, unaffected by the presence of asialofetuin (Fig. 6C) which could be explained by their high endocytic activity that is usually associated with endothelial cells.

4. Conclusion

Studies using fluorescent-labeled ligands for the ASGP-R have been carried out before. Ishihara et al.[42] prepared fluorescein isothiocyanate-labeled, galactosylated polystyrene ligands and analyzed their interaction with the ASGP-R by flow cytometry. Wu et al.[43] introduced a new synthetic route, based on solid phase peptide synthesis, toward fluorescent, synthetic, trivalent, *N*-acetylgalactosamine-terminated glycopeptides[43] as ligands for the ASGP-R.

However, in this study we have introduced a set of novel, fluorescent, trivalent, simplified oligosaccharide mimics as ligands for the ASGP-R (**6** and **7**, Fig. 3). These compounds not only comply with the afore-mentioned optimal ASGP-R ligand criteria, but also are synthetically easily accessible and hydrolytically stable.

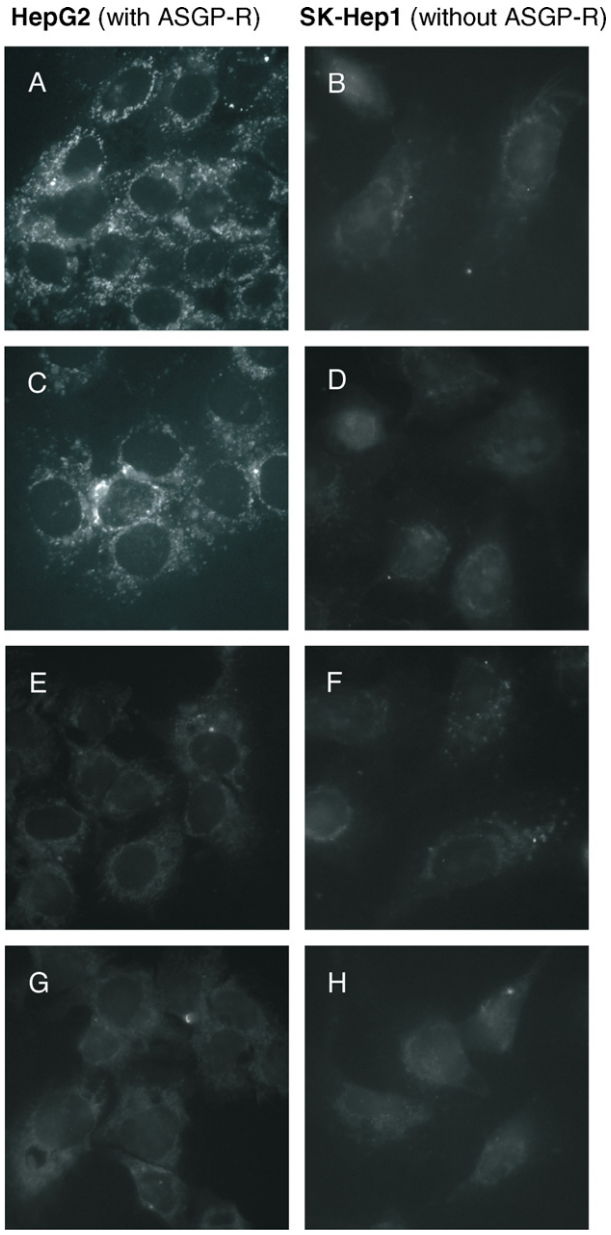


Figure 4. Fluorescence microscopy images depicting the ASGP-R-specific uptake of Alexa Fluor® 488-labeled compounds. (A) Compound **6** in HepG2 cells; (B) compound **6** with SK-Hep1 cells; (C) compound **7** in HepG2 cells; (D) compound **7** with SK-Hep1 cells; (E) compound **8** with HepG2 cells; (F) compound **8** with SK-Hep1 cells; (G) control HepG2 cells; (H) control SK-Hep1 cells.

Both criteria are a prerequisite for a therapeutic application at a later stage.

Moreover, using fluorescence microscopy and flow cytometry, we have shown that compounds **6** and **7** exhibit selective uptake by the ASGP-R on HepG2 cells derived from human parenchymal liver cells—the major liver cell type. The formation of distinct endocytic vesicles could be clearly visualized. Furthermore, competition with asialofetuin, a naturally occurring serum glycoprotein and known ligand of the ASGP-R, and Gal*N*Ac confirmed the involvement of the ASGP-R in the uptake of **7**. Experiments using compound **8** have further re-enforced the generally accepted assumption

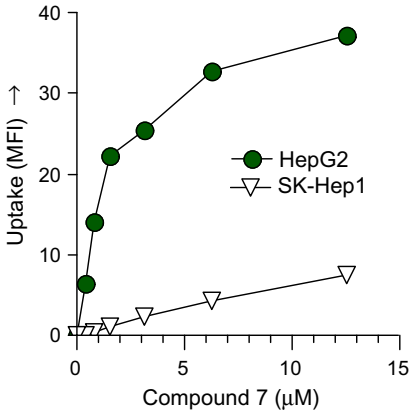


Figure 5. Titration of compound **7**: adherent HepG2 and SK-Hep1 cells were incubated with compound **7** at concentrations ranging from 0.4 to 12.5 μM for 40 min at 37 °C. MFI is the shift in median fluorescence intensity from untreated to treated cells.

that the sugar residues have to be in an optimal spatial arrangement in order to interact selectively and with high affinity with the native ASGP-R. In final analysis, we have demonstrated that compound **7** has a high potential for use in site-specific delivery of therapeutic agents (chemotherapeutics, DNA, etc.) to the liver. The follow-up experiments are currently being performed.

5. Experimental

5.1. General methods

NMR spectra were recorded on a Bruker Avance DMX-500 (500 MHz) spectrometer. Assignment of ^{1}H and ^{13}C NMR spectra was achieved using 2D methods (COSY, HSQC, TOCSY). Chemical shifts are expressed in parts per million using residual $CHCl_3$, CHD_2OD, and HDO as references. Optical rotations were measured using a Perkin-Elmer Polarimeter Model 341. ESI-MS spectra were measured on a Waters Micromass ZQ mass spectrometer. Reactions were monitored by TLC using glass plates coated with silica gel 60 F_{254} (Merck) with the following mobile phases: (A) petrol ether/EtOAc (4:1); (B) petrol ether/EtOAc (1:1); (C) petrol ether/EtOAc (3:7); (D) EtOAc; (E) EtOAc/MeOH (9:1); and (F) DCM/MeOH/H_2O (10:4:0.8). Carbohydrate-containing compounds were visualized by charring with a molybdate solution (0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4). Compounds **6**–**8** were visualized with UV light. All other compounds were visualized with $KMnO_4$ solution (2% $KMnO_4$ and 4% $NaHCO_3$ in water). Column chromatography was performed on silica gel 60 (Fluka, 0.040–0.060 mm). Size exclusion chromatography was performed on Sephadex LH-20 and Sephadex G-15 (Pharmacia). Methanol (MeOH) was dried by refluxing with sodium methoxide and distilled immediately before use. Pyridine was freshly distilled under argon over CaH_2. Dichloromethane (DCM) and dichloroethane (DCE) were dried by filtration over Al_2O_3 (Fluka, type 5016 A basic). Tetrahydro-

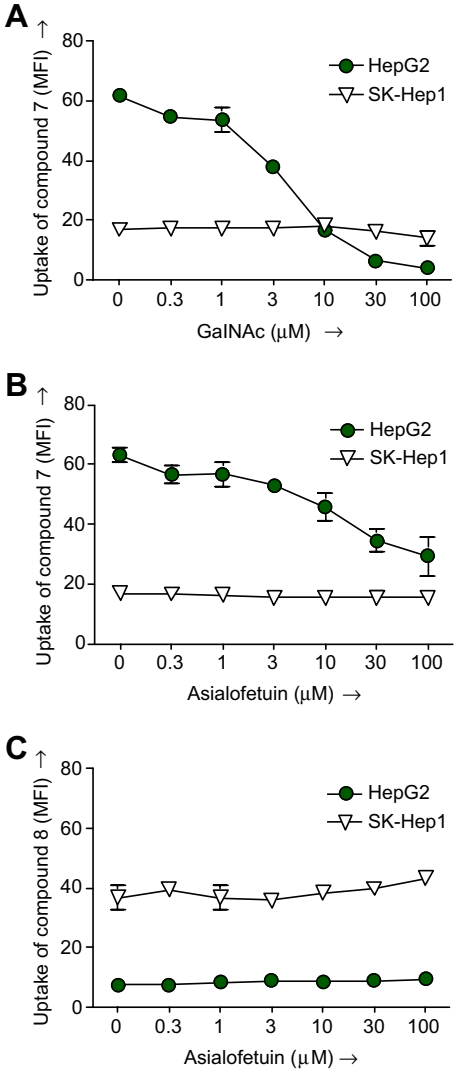


Figure 6. Competitive uptake of compound **7** at a concentration of 10 μM in the presence of either Gal*N*Ac (0.3–100 mM) (A) or asialofetuin (0.3–100 μM) (B). The graphs represent the mean of median fluorescence intensity (MFI) ±SD of three independent experiments. (C) The uptake of control compound **8** at a concentration of 10 μM in the presence of asialofetuin (0.3–100 μM) into HepG2 and ASGP-R-negative SK-Hep1 cells.

furan (THF), dioxane, diethyl ether (Et_2O), and toluene were dried by refluxing with sodium and benzophenone. Dry DMF was purchased from Fluka (absolute, $\geqslant$99.8%) and was further dried over powdered 4 Å molecular sieves. Molecular sieves (4 Å) were activated in vacuo at 500 °C for 2 h immediately before use. Alexa Fluor® 488 carboxylic acid succinimidyl ester (A20000, mixture of isomers) was purchased from Molecular Probes, Eugene, Oregon, USA. Zinc dust was activated according to the standard procedures.[44]

All cell culture media, supplements, and phosphate-buffered saline (PBS) were purchased from Invitrogen, except collagen type S from rat's tail was obtained from Roche Applied Science. Paraformaldehyde, NaN_3, and *N*-propyl gallate were obtained from Fluka. Bovine serum albumin (BSA) was from Sigma and Mowiol 4-88 from Hoechst. HepG2 (human hepatocellular carcinoma) and SK-Hep1 (human liver adenocarcinoma) cell lines were obtained from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen). Both cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) high-glucose, without phenol red, supplemented with fetal bovine serum (FBS, 10%) 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). During the incubation steps of the cells outside the incubator, medium with a CO_2-independent buffer system was used (DMEM high-glucose, without phenol red and FBS, containing 25 mM Hepes).

5.2. General procedure A: the preparation of triacetylated compounds 11, 13, and 15

To the corresponding triallylated compound (3.54 mmol) was added 9-BBN (0.5 M in THF, 38 ml) dropwise. The solution was then stirred at rt under argon for 24 h. The mixture was cooled to 0 °C, and aqueous NaOH (3 M, 39 ml) was added dropwise, followed by the dropwise addition of H_2O_2 (30%, 8.9 ml). The resultant mixture was stirred vigorously at rt for 24 h. The mixture was saturated with K_2CO_3, and the organic layer was separated. The aqueous layer was then extracted with THF (3× 80 ml), and the combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was dissolved in pyridine (33.5 ml), acetic anhydride (33.5 ml) was added, and the mixture was stirred at rt for 3 h. The mixture was co-evaporated with toluene (200 ml), and the resultant syrup was purified by silica gel chromatography to afford compound **11**, **13**, or **15** as oil.

5.2.1. Tris(5-acetoxy-2-oxapentyl)-*N*-(*tert*-butyloxycarbonyl)-methylamine (11). According to general procedure A, compound **10**[34] (1.21 g, 3.54 mmol) was reacted with 9-BBN (0.5 M in THF, 38 ml), and then treated with aqueous NaOH (3 M, 39 ml) and H_2O_2 (30%, 8.9 ml). After peracetylation, work-up, and chromatography on silica gel (petrol ether/EtOAc 8:2 → 7:3; R_f 0.52 B), 1.49 g (81%) of **11** was obtained. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.42 (s, 9H, CMe_3), 1.88 (m, 6H, 3× $OCH_2CH_2CH_2OAc$), 2.04 (s, 9H, 3× OAc), 3.50 (t, J = 6.1 Hz, 6H, 3× $OCH_2CH_2CH_2OAc$), 3.63 [s, 6H, $C(CH_2O)_3$], 4.13 (t, J = 6.5 Hz, 6H, 3× $OCH_2CH_2CH_2OAc$), 4.90 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 21.1 (3C, 3× CH_3C=O), 28.4 (3C, CMe_3), 28.9 (3C, 3× $OCH_2CH_2CH_2OAc$), 58.5 [$C(CH_2O)_3$], 61.7 (3C, 3× $OCH_2CH_2CH_2OAc$), 67.8 (3C, 3× $OCH_2CH_2CH_2OAc$), 69.5 [3C, $C(CH_2O)_3$], 79.1 (CMe_3), 154.8 [N–(C=O)O], 171.1 (3C, 3× CH_3C=O). Anal. Calcd for $C_{24}H_{43}NO_{11}$: C, 55.26; H, 8.31; N, 2.69. Found: C, 55.20; H, 8.24; N, 2.70.

5.2.2. Tris(9-acetoxy-2,6-dioxanonyl)-*N*-(*tert*-butyloxycarbonyl)-methylamine (13). According to general procedure A, compound **12** (1.82 g, 3.54 mmol) was reacted with 9-BBN (0.5 M in THF, 38 ml), and then treated with aqueous NaOH (3 M, 39 ml) and H_2O_2 (30%, 8.9 ml). After peracetylation, work-up, and chromatography on silica gel (petrol ether/EtOAc 3:1 → 3:2; R_f 0.32 B), 2.14 g (87%) of **13** was obtained. ^{1}H NMR (500 MHz,

$CDCl_3$): δ = 1.42 (s, 9H, CMe_3), 1.78–1.91 [m, 12H, 3× ($OCH_2CH_2CH_2)_2OAc$], 2.05 (s, 9H, 3× OAc), 3.45–3.50 (m, 18H, 3× $OCH_2CH_2CH_2OCH_2CH_2CH_2OAc$), 3.62 [s, 6H, $C(CH_2O)_3$], 4.15 (t, J = 6.5 Hz, 6H, 3× $OCH_2CH_2CH_2OAc$), 4.93 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 21.0 (3C, 3× CH_3C=O), 28.4 (3C, CMe_3), 29.0, 29.9 [6C, 3× ($OCH_2CH_2CH_2)_2OAc$], 58.4 [$C(CH_2O)_3$], 61.8 (3C, 3× $OCH_2CH_2CH_2OAc$), 67.2, 67.9, 68.3, (9C, 3× $OCH_2CH_2CH_2OCH_2CH_2CH_2OAc$), 69.5 [3C, $C(CH_2O)_3$], 79.1 (CMe_3), 154.8 [N–(C=O)O], 171.1 (3C, 3× CH_3C=O). Anal. Calcd for $C_{33}H_{61}NO_{14}$: C, 56.96; H, 8.84; N, 2.01. Found: C, 57.67; H, 8.80; N, 2.56.

5.2.3. Tris(13-acetoxy-2,6,10-trioxatridecyl)-*N*-(*tert*-butyloxycarbonyl)-methylamine (15). According to general procedure A, compound **14** (2.44 g, 3.54 mmol) was reacted with 9-BBN (0.5 M in THF, 38 ml), and then treated with aqueous NaOH (3 M, 39 ml) and H_2O_2 (30%, 8.9 ml). After peracetylation, work-up, and chromatography on silica gel (petrol ether/EtOAc 1:1 → 3:7; R_f 0.1 B) 2.71 g (88%) of **15** was obtained. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.39 (s, 9H, CMe_3), 1.75–1.88 [m, 18H, 3× ($OCH_2CH_2CH_2)_3OAc$], 2.01 (s, 9H, 3× OAc), 3.41–3.47 (m, 30H, 3× [($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OAc$]), 3.59 [s, 6H, $C(CH_2O)_3$], 4.12 (t, J = 6.5 Hz, 6H, 3× $OCH_2CH_2CH_2OAc$), 4.90 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.9 (3C, 3× CH_3C=O), 28.4 (3C, CMe_3), 28.9, 29.6, 29.9 [9C, 3× ($OCH_2CH_2CH_2)_3OAc$], 58.4 [$C(CH_2O)_3$], 61.7 (3C, 3× $OCH_2CH_2CH_2OAc$), 67.2, 67.7, 67.8, 67.9, 68.3, 68.4 [15C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OAc$], 69.4 [3C, $C(CH_2O)_3$], 78.8 (CMe_3), 154.7 [N–(C=O)O], 171.0 (3C, 3× CH_3C=O). Anal. Calcd for $C_{42}H_{79}NO_{17}$: C, 57.98; H, 9.15; N, 1.61; O, 31.26. Found: C, 58.08; H, 9.17; N, 1.70; O, 31.11.

5.3. General procedure B: preparation of triallylated compounds 12 and 14

The corresponding triacetylated compound (4.6 mmol) was dissolved in a solution of sodium methoxide in MeOH (0.1 M, 40 ml), and the resultant solution was stirred at rt for 4 h under argon. The solution was neutralized with Dowex 50X8 (H^+-form), and the solvent was removed under reduced pressure to afford the desired product in a quantitative yield as a colorless oil, which was used without further purification.

The corresponding triol (1 mmol) was then dissolved in DCM (5 ml) and the solution was added to a mixture of 50% aqueous NaOH (16 ml, w/v) and 15-crown-5 (19.8 μl, 0.1 mmol). Allyl bromide (1.64 ml, 19.1 mmol) was then added, and the resultant mixture was refluxed with vigorous stirring for 24 h. The mixture was cooled, and the DCM (top) layer was separated, dried with Na_2SO_4, and the solvent evaporated in vacuo. The resultant syrup was purified by silica gel chromatography to yield compound **12** or **14** as a yellow oil.

5.3.1. Tris(5-allyloxy-2-oxapentyl)-*N*-(*tert*-butyloxycarbonyl)-methylamine (12). According to general procedure B, compound **11** (2.39 g, 4.6 mmol) was deacetylated under Zemplén conditions, and after work-up, reacted with allyl bromide (7.5 ml, 87.9 mmol) under phase transfer catalysis conditions. After work-up and chromatography on silica gel (petrol ether/EtOAc 19:1 → 9:1 → 4:1; R_f 0.25 A), 2.25 g (95%) of **12** was obtained.^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.38 (s, 9H, CMe_3), 1.79 (quintet, J = 6.3 Hz, 6H, 3× $OCH_2CH_2CH_2O$), 3.43–3.48 (m, 12H, 3× $OCH_2CH_2CH_2O$), 3.59 [s, 6H, $C(CH_2O)_3$], 3.91 (m, 6H, 3× CH_2CH=CH_2), 4.90 (s, 1H, NH), 5.17 (m, 6H, 3× CH_2CH=CH_2), 5.86 (m, 3H, 3× CH_2CH=CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 28.4 (3C, CMe_3), 30.0 (3C, 3× $OCH_2CH_2CH_2O$), 58.5 [$C(CH_2O)_3$], 67.4, 68.2 (6C, 3× $OCH_2CH_2CH_2O$), 69.6 [3C, $C(CH_2O)_3$], 71.9 (3C, 3× CH_2CH=CH_2), 78.8 (CMe_3), 116.7 (3C, 3× CH_2CH=CH_2), 135.0 (3C, 3× CH_2CH=CH_2), 154.8 [N–(C=O)O]. Anal. Calcd for $C_{27}H_{49}NO_8$: C, 62.89; H, 9.58; N, 2.72; O, 24.82. Found: C, 62.66; H, 9.61; N, 2.68; O, 24.87.

5.3.2. Tris(9-allyloxy-2,6-dioxanonyl)-*N*-(*tert*-butyloxycarbonyl)-methylamine (14). According to general procedure B, compound **13** (3.2 g, 4.6 mmol) was deacetylated under Zemplén conditions, and after work-up, reacted with allyl bromide (7.5 ml, 87.9 mmol) under phase transfer catalysis conditions. After work-up and chromatography on silica gel (petrol ether/EtOAc 4:1 → 1:1; R_f 0.64 B), 2.86 g (90%) of **14** was obtained. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.42 (s, 9H, CMe_3), 1.78–1.87 (m, 12H, 6× $OCH_2CH_2CH_2O$), 3.45–3.52 (m, 24H, 6× $OCH_2CH_2CH_2O$), 3.62 [s, 6H, $C(CH_2O)_3$], 3.96 (m, 6H, 3× CH_2CH=CH_2), 4.93 (s, 1H, NH), 5.22 (m, 6H, 3× CH_2CH=CH_2), 5.91 (m, 3H, 3×CH_2CH=CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 28.4 (3C, CMe_3), 29.9, 30.1 (6C, 6× $OCH_2CH_2CH_2O$), 58.4 [$C(CH_2O)_3$], 67.3, 67.8, 68.4 (12C, 6× $OCH_2CH_2CH_2O$), 69.5 [3C, $C(CH_2O)_3$], 71.8 (3C, 3× CH_2CH=CH_2), 79.0 (CMe_3), 116.7 (3C, 3× CH_2CH=CH_2), 134.9 (3C, 3× CH_2CH=CH_2), 155.0 [N–(C=O)O]. Anal. Calcd for $C_{36}H_{67}NO_{11}$: C, 62.67; H, 9.79; N, 2.03; O, 25.51. Found: C, 62.67; H, 9.72; N, 2.10; O, 25.37.

5.4. Tris(13-acetoxy-2,6,10-trioxatridecyl)-methylamine hydrochloride (16)

Compound **15** (680 mg, 0.781 mmol) was dissolved in 4 M HCl in dioxane (10 ml), and the resultant mixture was stirred at rt under argon for 30 min. The solvent was removed in vacuo to yield **16** (630 mg, quantitative) as an oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.68–1.77 [m, 18H, 3× ($OCH_2CH_2CH_2)_3OAc$], 1.92 (s, 9H, 3× OAc), 3.35–3.44 [m, 36H, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OAc$, $C(CH_2O)_3$], 4.01 (t, J = 6.5 Hz, 6H, 3× $OCH_2CH_2CH_2OAc$); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.5 (3C, 3× CH_3C=O), 28.6, 29.3, 29.6 [9C, 3× ($OCH_2CH_2CH_2)_3OAc$], 59.1 [$C(CH_2O)_3$], 61.6 (3C, 3× $OCH_2CH_2CH_2OAc$), 66.7, 67.0, 67.4, 67.6, 68.3, 68.7 [18C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OAc$, $C(CH_2O)_3$],171.5 (3C, 3× CH_3C=O); ESI-MS: Calcd for $C_{37}H_{72}NO_{15}$ $(M+H)^+$: 770.49. Found: *m/z* 770.54.

5.5. *N*-{Tris[13-acetoxy-2,6,10-trioxatridecyl]methyl}-4-(benzyloxycarbonyl-amino)-butyramide (18)

Compound **16** (239 mg, 0.297 mmol), **17**[36] (70.4 mg, 0.297 mmol), and PyBOP (186 mg, 0.357 mmol) were dissolved in dioxane/DMF (4 ml, 3:1 v/v), and DIPEA (229 μl, 1.34 mmol) was added. The mixture was stirred at rt under argon for 24 h. The resultant solution was partitioned between DCM (15 ml) and H_2O (15 ml). The DCM layer was separated, and the aqueous phase was extracted with DCM (25 ml). The DCM fractions were combined, dried (Na_2SO_4), and the solvent was removed under reduced pressure. The resultant syrup was purified by silica gel chromatography (petrol ether/EtOAc 1:1 → 3:7 → 0:1) to afford **18** (250 mg, 85%, R_f 0.22 D) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.76–1.90 (m, 20H, 3× [$(OCH_2CH_2CH_2)_3OAc$, NCH_2CH_2-$CH_2C{=}O$]), 2.03 (s, 9H, 3× OAc), 2.07–2.19 (m, 3H, $NCH_2CH_2CH_2C{=}O$), 3.20–3.24 (m, 3H, NCH_2-$CH_2CH_2C{=}O$), 3.41–3.50 [m, 30H, 3× (OCH_2CH_2-$CH_2)_2OCH_2CH_2CH_2OAc$], 3.66 [s, 6H, $C(CH_2O)_3$], 4.13 (t, J = 6.5 Hz, 6H, 3× $OCH_2CH_2CH_2OAc$), 5.07 (s, 2H, CH_2Ph), 5.28 (br s, 1H, NH, Cbz), 5.86 (s, 1H, NH, Tris), 7.28–7.34 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.9 (3C, 3× $CH_3C{=}O$), 25.8 (1C, NCH_2-$CH_2CH_2C{=}O$), 29.0, 29.8, 30.1 [9C, 3× (OCH_2CH_2-$CH_2)_3OAc$], 34.4 (1C, $NCH_2CH_2CH_2C{=}O$), 40.3 (1C, $NCH_2CH_2CH_2C{=}O$), 59.8 [$C(CH_2O)_3$], 61.8 (3C, 3× $OCH_2CH_2CH_2OAc$), 66.5 (1C, CH_2Ph), 67.3, 67.7, 67.9, 68.4 [15C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2$-$CH_2OAc$], 69.1 [3C, $C(CH_2O)_3$], 128.0, 128.1, 128.5, 136.7 (6C, C_6H_5), 156.6 [N-(C=O)O], 171.1 (3C, 3× $CH_3C{=}O$), 172.4 (C=O, amide). Anal. Calcd for $C_{49}H_{84}N_2O_{18}$: C, 59.50; H, 8.56; N, 2.83; O, 29.11. Found: C, 59.12; H, 8.36; N, 2.98; O, 29.56.

5.6. 4-(Benzyloxycarbonylamino)-*N*-{tris[13-hydroxy-2,6,10-trioxatridecyl]methyl}-butyramide (19)

Compound **18** (231 mg, 0.233 mmol) was dissolved in a solution of sodium methoxide in dry methanol (0.05 M, 20 ml), and the resultant solution was stirred at rt under argon for 4 h. The reaction mixture was neutralized with Dowex 50X8 (H^+-form), and the solvent was removed in vacuo. The resultant oil was purified by silica gel chromatography (EtOAc/MeOH 95:5 → 9:1) to afford **19** (181 mg, 90%, R_f 0.2 E) as an oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.76–1.83 [m, 20H, 3× ($OCH_2CH_2CH_2)_3OH$, $NCH_2CH_2CH_2C{=}O$], 2.18 (t, J = 6.8 Hz, 2H, NCH_2-$CH_2CH_2C{=}O$), 2.62 (bs, 3H, 3× OH), 3.21 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.43–3.51 (m, 24H, 3× OCH_2CH_2-$CH_2OCH_2CH_2CH_2OCH_2CH_2CH_2OH$), 3.58 [t, J = 5.8 Hz, 6H, 3× $OCH_2CH_2CH_2(OCH_2CH_2CH_2)_2OH$], 3.67 [s, 6H, $C(CH_2O)_3$], 3.73 (t, J = 5.7 Hz, 6H, 3× $OCH_2CH_2CH_2OH$), 5.07 (s, 2H, CH_2Ph), 5.45 (s, 1H, NH, Cbz), 6.00 (s, 1H, NH, Tris), 7.28–7.34 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 25.7 ($NCH_2CH_2CH_2C{=}O$), 29.8, 30.0, 32.0 [9C, 3× ($OCH_2CH_2CH_2)_3OH$], 34.3 ($NCH_2CH_2CH_2C{=}O$), 40.3 ($NCH_2CH_2CH_2C{=}O$), 59.9 [$C(CH_2O)_3$], 61.7 (3C, 3× $OCH_2CH_2CH_2OH$), 66.5 (CH_2Ph), 67.7, 68.1, 68.3 (12C, 3× $OCH_2CH_2CH_2OCH_2CH_2CH_2OCH_2CH_2CH_2$-$OH$), 69.2 [3C, $C(CH_2O)_3$], 69.7 [3C, 3× OCH_2CH_2-$CH_2(OCH_2CH_2CH_2)_2OH$], 128.0, 128.1, 128.5, 136.7 (6C, C_6H_5), 156.7 [N–(C=O)O], 173.5 (C=O, amide); ESI-MS: Calcd for $C_{43}H_{78}N_2O_{15}Na$ $(M+Na)^+$: 885.53. Found: *m*/*z* 885.68.

5.7. *N*-{Tris[13-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyloxy)-2,6,10-trioxatridecyl]methyl}-(4-benzyloxycarbonylamino)-butyramide (21)

Compound **19** (151 mg, 0.173 mmol) and ethyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-galactopyranoside (**20**)[27] (670 mg, 1.04 mmol) were dissolved in dry DCM (10 ml), and the mixture was stirred with 4 Å molecular sieves (500 mg) at rt under argon for 2 h. The mixture was cooled to 0 °C, and DMTST (538 mg, 2.08 mmol) was added. The reaction was stirred at 0 °C for 24 h, and then at 10 °C for another 24 h under argon. The mixture was then filtered and extracted with aqueous $NaHCO_3$ solution (10 ml, 1 M) and brine (10 ml). The organic phase was dried (Na_2SO_4), and the solvent was removed under reduced pressure. The resultant syrup was purified by silica gel chromatography (EtOAc/petrol ether 1:1 → 7:3 → 1:0) to afford the desired product **21** (301 mg, 68%, R_f 0.15C) as a colorless solid. $[\alpha]_D$ +72.9 (*c* 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.64–1.69, 1.76–1.85 [m, 20H, 3× ($OCH_2CH_2CH_2)_3OGal$, $NCH_2CH_2CH_2C{=}O$], 2.16 (m, 2H, NCH_2CH_2 $CH_2C{=}O$), 3.18 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.22–3.47 [m, 30H, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGal$], 3.66, [s, 6H, $C(CH_2O)_3$], 3.68, 4.01–4.06 (m, 6H, 3× $OCH_2CH_2CH_2OGal$), 4.32 (m, 3H, 3× H5-Gal), 4.40 (dd, $J_{5,6}$ = 6.7, $J_{6,6'}$ = 11.3 Hz, 3H, 3× H6-Gal), 4.68 (dd, $J_{5,6'}$ = 6.4, $J_{6,6'}$ = 11.2 Hz, 3H, 3× H6′-Gal), 4.81 (d, $J_{1,2}$ = 7.9 Hz, 3H, 3× H1-Gal), 5.07 (s, 2H, CH_2Ph), 5.27 (br s, 1H, NH, Cbz), 5.61 (m, 3H, 3× H3-Gal), 5.78 (m, 3H, 3× H2-Gal), 5.86 (s, 1H, NH, Tris), 5.99 (m, 3H, 3× H4-Gal), 7.22-7.26, 7.28–7.35, 7.37–8.09 (m, 65H, 13× C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 26.6 ($NCH_2CH_2CH_2C{=}O$), 29.7, 29.8, 29.9 [9C, 3× ($OCH_2CH_2CH_2)_3OGal$], 35.1 ($NCH_2CH_2CH_2C{=}O$), 42.2 ($NCH_2CH_2CH_2C{=}O$), 59.8 [$C(CH_2O)_3$], 61.9 (3C, 3× C6-Gal), 67.0 (CH_2Ph), 67.4, 67.8, 67.9 [12C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGal$], 68.1 (3C, 3× C4-Gal), 68.4 (3C, 3× $OCH_2CH_2CH_2OGal$), 69.1 [3C, $C(CH_2O)_3$], 69.8 (3C, 3× C2-Gal), 71.2 (3C, 3× C3-Gal), 71.7 (3C, 3× C5-Gal), 101.8 (3C, 3× C1-Gal), 128.0–136.7 (78C, C_6H_5), 165.2–166.0 (13C, 13 C=O). Anal. Calcd for $C_{145}H_{156}N_2O_{42}$: C, 67.01; H, 6.05; N, 1.08. Found: C, 66.51; H, 6.12; N, 1.13.

5.8. 4-(Benzyloxycarbonylamino)-*N*-{tris[13-(β-D-galactopyranosyloxy)-2,6,10-trioxatridecyl]methyl}-butyramide (5a)

Compound **21** (30 mg, 0.015 mmol) was dissolved in dry dioxane (1 ml), and a solution of sodium methoxide in methanol (0.1 M, 1 ml) was added. The resultant mixture was stirred at rt under argon for 4 h, after which it was neutralized with Dowex 50X8 (H^+-form), filtered, and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (DCM/MeOH/H_2O 10:3:0 → 10:4:0 → 10:4:0.1 → 10:4:0.2 → 10:4:0.4) to afford **5a** (14.6 mg, 94%, R_f 0.2

F) as a colorless solid. $[\alpha]_D$ −5.73 (*c* 0.96, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 1.73–1.88 [m, 20H, 3× ($OCH_2CH_2CH_2)_3OGal$, $NCH_2CH_2CH_2C{=}O$], 2.19 (t, *J* = 7.3 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.15 (t, *J* = 6.8 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.44–3.75 [m, 54H, 3× H2-Gal, 3× H3-Gal, 3× H5-Gal, 3× H6-Gal, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGal$, $C(CH_2O)_3$, 3× $OCH_2CH_2CH_2OGal$-H_a], 3.82 (m, 3H, 3× H4-Gal), 3.95 (m, 3H, 3× $OCH_2CH_2CH_2OGal$-H_b), 4.20 (d, $J_{1,2}$ = 7.4 Hz, 3H, 3× H1-Gal), 5.07 (s, 2H, CH_2Ph), 7.29–7.35 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, MeOD): δ = 27.5 ($NCH_2CH_2CH_2C{=}O$), 31.0, 31.1 [9C, 3× ($OCH_2CH_2CH_2)_3OGal$], 35.0 ($NCH_2CH_2CH_2C{=}O$), 41.1 ($NCH_2CH_2CH_2C{=}O$), 61.6 [$C(CH_2O)_3$], 62.4 (3C, 3× C6-Gal), 67.4 (CH_2Ph), 67.9 (3C, 3× $OCH_2CH_2CH_2OGal$), 68.8 (3C, 3× $C[CH_2O]_3$), 69.4 (3C, 3× C4-Gal), 68.8, 70.2, 72.6, 75.0, 76.6 [24C, 3× C2-Gal, 3× C3-Gal, 3× C5-Gal, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGal$], 105.1 (3C, 3× C1-Gal), 128.9–129.5, 138.4 (6C, C_6H_5), 158.9 [N–(C=O)O], 175.6 (C=O); ESI-MS: Calcd for $C_{61}H_{109}N_2O_{30}$ $(M+H)^+$: 1349.71. Found: *m*/*z* 1349.87.

5.9. 4-Amino-*N*-{tris[13-(β-D-galactopyranosyloxy)-2,6,10-trioxatridecyl]methyl}-butyramide (22)

Compound **5a** (25 mg, 18.5 μmol) was dissolved in ethanol/dioxane (2 ml, 1:1 v/v), and Pd/C (10% Pd, 20 mg) was added. The mixture was vigorously stirred under a H_2 atmosphere (1 atm) at rt for 24 h. The mixture was then diluted with ethanol, filtered, and concentrated in vacuo to yield **22** as a colorless solid (19.5 mg, 87%). $[\alpha]_D$ −5.8 (*c* 1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 1.79–1.89 [m, 20H, 3× ($OCH_2CH_2CH_2)_3OGal$, $NCH_2CH_2CH_2C{=}O$], 2.31 (t, *J* = 7.1 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 2.87 (t, *J* = 7.3 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.44–3.56 [m, 39H, 3× H2-Gal, 3× H3-Gal, 3× H5-Gal, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGal$], 3.60–3.75 (m, 15H, 3× H6-Gal, $C[CH_2O]_3$, 3× $OCH_2CH_2CH_2OGal$-H_a), 3.82 (m, 3H, 3× H4-Gal), 3.96, (m, 3H, 3× $OCH_2CH_2CH_2OGal$-H_b), 4.2 (d, $J_{1,2}$ = 7.2 Hz, 3H, 3× H1-Gal); ^{13}C NMR (125 MHz, MeOD): δ = 26.9 ($NCH_2CH_2CH_2C{=}O$), 30.7, 31.0, 31.1 [9C, 3× ($OCH_2CH_2CH_2)_3Gal$], 34.6 ($NCH_2CH_2CH_2C{=}O$), 48.3 ($NCH_2CH_2CH_2C{=}O$), 62.5 (3C, 3× C6-Gal), 67.9, 68.8, 68.9, 69.4, 69.7 [21C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGal$, 3× $OCH_2CH_2CH_2OGal$, $C(CH_2O)_3$], 70.2 (3C, 3× C4-Gal), 72.6 (3C, 3× C2-Gal), 75.0 (3C, 3× C3-Gal), 76.6 (3C, 3× C5-Gal), 105.1 (3C, 3× C1-Gal); ESI-MS: Calcd for $C_{53}H_{103}N_2O_{28}$ $(M+H)^+$: 1215.67. Found: *m*/*z* 1215.91.

5.10. Fluorescent-labeled, Gal-terminated compound (6)

A stock solution containing compound **22** (10 mg, 8.23 μmol), DIPEA (20 μl, 156 μmol) and 4 Å molecular sieves (25 mg) in dry DMF/dioxane (1 ml, 1:1) was stirred at rt under argon for 2 h. The solution (500 μl) was transferred to a small vial containing Alexa Fluor® 488-NHS (1 mg, 1.55 μmol), 4 Å molecular sieves (25 mg), and a stirring bar. The resultant mixture was stirred in the dark at rt under argon for 4 days. The mixture was then diluted with MeOH, filtered, and the solvents were removed in vacuo. The residue was purified by gel filtration on a Sephadex LH-20 column (2.5 × 35 cm) using MeOH as an eluant, then on an RP-18 column (H_2O/MeOH stepwise gradient 1:0–1:1) to yield **6** (2.2 mg, 81%) as a red solid after final lyophilization from water. ESI-MS: Calcd for $C_{74}H_{112}N_4O_{38}S_2^{2-}(M/2)^-$: 864.32. Found: *m*/*z* 864.85.

5.11. *N*-(Tris{13-[3,4,6-tri-*O*-acetyl-2-(2,2,2-trichloroethoxycarbonylamino)-β-D- galactopyranosyloxy]-2,6,10-trioxatridecyl}methyl)-(4-benzyloxycarbonyl-amino)-butyramide (24)

Compound **19** (15 mg, 17.3 μmol) and ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-galactopyranoside (**23**)[37] (54.6 mg, 104 μmol) were dissolved in dry DCM (1 ml) and stirred with 4 Å molecular sieves (50 mg) at rt under argon for 2 h. The suspension was cooled to 0 °C, and DMTST (53.8 mg, 208 μmol) was added. The mixture was stirred at 0 °C for 24 h, then at 10 °C for 48 h. The reaction was quenched with triethylamine (50 μl), diluted with DCM (5 ml), washed with brine (5 ml), dried (Na_2SO_4), and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (petrol ether/EtOAc 1:4 → 1:9) to afford **24** (35.1 mg, 91%, R_f 0.32 D) as a colorless solid. $[\alpha]_D$ −3.6 (*c* 1.76, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.76–1.84 [m, 20H, 3× ($OCH_2CH_2CH_2)_3OGalN$, $NCH_2CH_2CH_2C{=}O$], 1.96 (s, 9H, 3× OAc), 2.03 (s, 9H, 3× OAc), 2.14 (s, 9H, 3× OAc), 2.20 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.22 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.43–3.56 [m, 30H, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGalN$], 3.60 (m, 3H, 3× CH_2OGalN-H_a), 3,66, [s, 6H, $C(CH_2O)_3$], 3.85–3.90 (m, 3× H2-GalN, 6H, 3× H5-GalN), 3.97 (m, 3H, 3× CH_2OGalN-H_b), 4.05–4.19 (m, 6H, 3× H6-GalN, 3× H6′-GalN), 4.56 (d, $J_{1,2}$ = 7.7 Hz, 3H, 3× H1-GalN), 4.66, 4.75 (A, B of AB, *J* = 11.7 Hz, 6H, 3× CH_2, Troc), 5.08 (s, 2H, CH_2Ph), 5.14 (m, 3H, 3× H3-GalN), 5.35 (m, 3H, 3× H4-GalN), 5.78 (d, *J* = 7.6 Hz, 3H, N–H, GalN), 5.95 (br s, 1H, NH, Tris), 7.32 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.6, 20.7, 20.8 (9C, 9× CH_3, AcO), 25.8 ($NCH_2CH_2CH_2C{=}O$), 29.6, 29.7, 29.8 [9C, 3× ($OCH_2CH_2CH_2)_3OGalN$], 34.8 ($NCH_2CH_2CH_2C{=}O$), 40.7 ($NCH_2CH_2CH_2C{=}O$), 59.8 [$C(CH_2O)_3$], 61.4 (3C, 3× C6-GalN), 66.6 (CH_2Ph), 66.9 (3C, 3× C4-GalN), 67.4, 67.5, 67.6 [15C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2OGalN$], 68.4 (3C, 3× $OCH_2CH_2CH_2OGalN$), 69.1 [3C, $C(CH_2O)_3$], 70.1 (3C, 3× C3-GalN), 70.5 (3C, 3× C5-GalN), 95.6 (3C, 3× CCl_3, Troc), 101.6 (3C, 3× C1-GalN), 128.0–128.5 (6C, C_6H_5), 154.3, 154.5 [4C, 4× N–(C=O)O], 170.3–171.2 (10C, 10× C=O). Anal. Calcd for $C_{88}H_{132}Cl_9N_5O_{42}$: C, 46.95; H, 5.91; N, 3.11. Found: C, 47.22; H, 6.00; N, 3.03.

5.12. *N*-{Tris[13-(2-acetamido-3,4,6-tri-*O*-acetyl-β-D-galactopyranosyloxy)-2,6,10-trioxatridecyl]methyl}-(4-benzyloxycarbonylamino)-butyramide (25)

Compound **24** (20 mg, 8.88 μmol) was dissolved in dry dioxane (1 ml), and activated Zn dust (55 mg, 84.1 mmol) was added, followed by acetic anhydride (272 μl, 2.66 mmol), and the reaction mixture was stirred at rt un-

der argon for 16 h. The mixture was filtered and the solvents were removed in vacuo. The residue was purified by silica gel chromatography (DCM/MeOH stepwise gradient 99:1 → 93:7) to afford **25** (13.5 mg, 82%, R_f 0.13 E) as a colorless solid. $[\alpha]_D$ −12.2 (*c* 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.76–1.85 [m, 20 H, 3× ($OCH_2CH_2CH_2)_3$OGalN, $NCH_2CH_2CH_2C{=}O$], 1.95 (s, 9H, 3× NAc), 1.99, 2.04, 2.14 (s, 27H, 9× OAc), 2.19 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.22 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.44–3.48 [m, 30H, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2$OGalN], 3.58 (m, 3H, 3× CH_2O-GalN-H_a), 3,67 [s, 6H, $C(CH_2O)_3$], 3.89–3.96 (m, 6H, 3× H5-GalN, 3×CH_2OGalN-H_b), 4.00 (m, 3H, 3× H2-GalN), 4.09–4.18 (m, 6H, 3× H6-GalN, 3× H′-GalN), 4.64 (d, $J_{1,2}$ = 8.3 Hz, 3H, 3× H1-GalN), 5.08 (s, 2H, CH_2Ph), 5.23 (m, 3H, 3× H3-GalN), 5.34 (s, 3H, 3× H4-GalN), 5.41 (m, 1H, NH, Cbz), 6.05 (d, *J* = 8.6 Hz, 3H, NHAc), 7.30–7.34 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.6 (9C, 9× CH_3, AcO), 23.3 (3C, 3× CH_3, AcHN), 25.9 (1C, $NCH_2CH_2CH_2C{=}O$), 29.9, 29.8, 29.6 [9C, 3× ($OCH_2CH_2CH_2)_3$OGalN], 34.5 ($NCH_2CH_2CH_2C{=}O$), 40.3 ($NCH_2CH_2CH_2C{=}O$), 51.3 (3C, 3× C2-GalN), 59.8 [$C(CH_2O)_3$], 61.4 (3C, 3× C6-GalN), 66.5 (CH_2Ph), 66.7 (3C, 3× C4-GalN), 67.0 (3C, 3× $OCH_2CH_2CH_2$OGalN), 67.7 [15C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2$OGalN], 69.0 [3C, $C(CH_2O)_3$], 70.0 (3C, 3× C3-GalN), 70.4 (3C, 3× C5-GalN), 101.2 (3C, 3× C1-GalN), 127.9, 128.0, 128.5,136.6 (6C, C_6H_5), 156.7, [1C,N–(C=O)O] 170.2–172.5, (13C, 13× C=O). Anal. Calcd for $C_{85}H_{135}N_5O_{39}$: C, 55.15; H, 7.35; N, 3.78. Found: C, 54.84; H, 7.34; N, 3.61.

5.13. *N*-{Tris[13-(2-acetamido-β-D-galactopyranosyloxy)-2,6,10-trioxatridecyl]methyl}-4-(benzyloxycarbonylamino)-butyramide (5b)

Compound **25** (35 mg, 18.9 μmol) was dissolved in dry dioxane (1 ml), and a solution of sodium methoxide in methanol (0.1 M, 1 ml) was added. The mixture was stirred at rt under argon for 4 h, after which it was neutralized with Dowex 50X8 (H^+-form), filtered, and the solvent was removed under reduced pressure. The residue was purified on an RP-18 column (H_2O/MeOH, stepwise gradient, 1:0 → 2:3) to afford **5b** (20.1 mg, 72%, R_f 0.1 F) as a colorless solid. $[\alpha]_D$ −1.7 (*c* 1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 1.75–1.82 [m, 20H, 3× ($OCH_2CH_2CH_2)_3$O-GalN, $NCH_2CH_2CH_2C{=}O$], 1.99 (s, 9H, 3× NAc), 2.20 (t, *J* = 7.3 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.16 (t, *J* = 6.8 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.47–3.49 [m, 33H, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2$OGalN, 3× H5-GalN], 3.54–3.61 (m, 6H, 3× CH_2OGalN-H_a, 3× H3-GalN), 3.67 [s, 6H, $C(CH_2O)_3$], 3.72–3.79 (m, 6H, 3× H6-GalN), 3.83 (m, 3H, 3× H4-GalN), 3.89–3.95 (m, 6H, 3× CH_2OGalN-H_b, 3× H2-GalN), 4.36 (d, $J_{1,2}$ = 8.4 Hz, 3H, 3× H1-GalN), 5.07 (s, 2H, CH_2Ph), 7.24–7.35 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, MeOD): δ = 23.1 (3C, 3× CH_3, AcHN), 27.6 ($NCH_2CH_2CH_2C{=}O$), 31.0, 30.1 [9C, 3× ($OCH_2CH_2CH_2)_3$OGalN], 35.0 ($NCH_2CH_2CH_2C{=}O$), 41.1 ($NCH_2CH_2CH_2C{=}O$), 54.3 (3C, 3× C2-GalN), 61.6 [$C(CH_2O)_3$], 62.5 (3C, 3× C6-GalN), 67.4 (4C, 3× $OCH_2CH_2CH_2$OGalN, CH_2Ph), 68.9, 68.8, 68.6 [15C, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2$OGalN], 66.4 (3C, 3× C4-GalN), 69.6 [3C, $C(CH_2O)_3$], 73.3 (3C, 3× C3-GalN), 76.6 (3C, 3× C5-GalN), 103.1 (3C, 3× C1-GalN), 128.8, 128.9, 129.5, 138.4 (6C, C_6H_5), 158.9 [N–(C=O)O]; ESI-MS: Calcd for $C_{67}H_{117}N_5O_{30}Na$ $(M+Na)^+$: 1494.77. Found: *m*/*z* 1495.41.

5.14. *N*-{Tris[13-(2-acetamido-β-D-galactopyranosyloxy)-2,6,10-trioxatridecyl]methyl}-4-amino-butyramide (26)

Compound **5b** (15 mg, 10.2 μmol) was dissolved in methanol/dioxane (2 ml, 1:1 v/v), and Pd/C (10% Pd, 15 mg) was added. The resultant suspension was vigorously stirred under a H_2 atmosphere (1 atm) at rt for 3 h. The mixture was then diluted with methanol, filtered, and concentrated in vacuo to yield **26** as a colorless solid (13 mg, 95%). $[\alpha]_D$ −1.49 (*c* 0.67, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 1.78–1.82 [m, 20H, 3× ($OCH_2CH_2CH_2O)_3$GalN, $NCH_2CH_2CH_2C{=}O$], 1.99 (s, 9H, 3× NAc), 2.29 (t, *J* = 7.2 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 2.82 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.48–3.51 [m, 33H, 3× ($OCH_2CH_2CH_2)_2OCH_2CH_2CH_2$O-GalN, 3×H5-GalN], 3.55–3.63 (m, 6H, 3× CH_2OGalN-H_a, 3× H3-GalN), 3.67 [s, 6H, $C(CH_2O)_3$], 3.70–3.79 (m, 6H, 3×H6-GalN), 3.84 (m, 3H, 3× H4-GalN), 3.89–3.95 (m, 6H, 3× CH_2OGalN-H_b, 3× H2-GalN), 4.35 (d, $J_{1,2}$ = 8.4 Hz, 3H, 3× H1-GalN); ^{13}C NMR (125 MHz, MeOD): δ = 23.1 (3C, 3× CH_3, AcHN), 27.5 (1C, $NCH_2CH_2CH_2C{=}O$), 31.0, 30.1 [9C, 3× ($OCH_2CH_2CH_2)_3$OGalN], 34.7 ($NCH_2CH_2CH_2C{=}O$), 41.2 ($NCH_2CH_2CH_2C{=}O$), 54.3 (3C, 3× C2-GalN), 61.6 [$C(CH_2O)_3$], 62.5 (3C, 3× C6-GalN), 67.4 (3C, 3× $OCH_2CH_2CH_2$OGalN), 68.6, 68.8, 68.9 [15C, 3× ($OCH_2CH_2CH_{22}OCH_2CH_2CH_2$OGalN), 69.4 (3C, 3× C4-GalN]), 69.7 [3C, $C(CH_2O)_3$], 73.3 (3C, 3× C3-GalN), 76.6 (3C, 3× C5-GalN), 103.1 (3C, 3× C1-GalN), 174.0, 175.2 (4× C=O); ESI-MS: Calcd for $C_{59}H_{112}N_5O_{28}$ $(M+H)^+$: 1338.75. Found: *m*/*z* 1339.22.

5.15. Fluorescent-labeled, GalNAc-terminated compound (7)

A stock solution containing compound **26** (10 mg, 7.47 μmol), DIPEA (50 μl, 292 μmol), and 4 Å molecular sieves (25 mg) in dry DMF/dioxane (1 ml, 1:1) was stirred at rt under argon for 2 h. The resultant solution (500 μl) was transferred to a small vial containing Alexa Fluor® 488-NHS (1 mg, 1.55 μmol), 4 Å molecular sieves (25 mg), and a stirring bar. The mixture was stirred in the dark at rt under argon for 4 days, then diluted with MeOH, filtered, and the solvents were removed in vacuo. The residue was purified by gel filtration on a Sephadex LH-20 column (2.5 × 35 cm) using MeOH as an eluant, then on an RP-18 column (H_2O/MeOH stepwise gradient 1:0→1:1) to yield **7** (2.6 mg, 90%). ESI-MS: Calcd for $C_{80}H_{121}N_7O_{38}S_2^{2-}$ $(M/2)^-$: 925.86. Found: *m*/z 926.05.

5.16. 4-(Benzyloxycarbonylamino)-*N*-{[tris(hydroxymethyl)]methyl}-butyramide (27)

Compound **17**[36] (3.01 g, 12.6 mmol) and EEDQ (3.26 g, 13.2 mmol) were stirred in dry pyridine (100 ml) at rt under argon for 1 h. Tris (**9**) (1.33 g, 10.9 mmol) was then added, and the resultant suspension was stirred at 90 °C for 24 h. The solvent was removed in vacuo, and the res-

idue was triturated with EtOAc and a few drops of MeOH. The product was filtered off, washed with EtOAc, then with cold water, and dried under high vacuum to yield **27** (2.88 g, 77%) as a white solid. ^{1}H NMR (500 MHz, MeOD): δ = 1.79 (quintet, J = 7.0 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 2.28 (t, J = 7.4 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.16 (t, J = 6.8 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.73 (s, 6H, $C[CH_2O]_3$), 5.09 (m, 2H, CH_2Ph), 7.28–7.35 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 27.1 ($NCH_2CH_2CH_2C{=}O$), 34.5 ($NCH_2CH_2CH_2C{=}O$), 41.0 ($NCH_2CH_2CH_2C{=}O$), 62.5 [3C, $C(CH_2O)_3$], 63.6 [$C(CH_2O)_3$], 67.4 (CH_2Ph), 128.8, 129.0, 129.4, 130.6, 138.3 (6C, C_6H_5), 159.0 [N–(C=O)O], 176.6 (C=O); ESI-MS: Calcd for $C_{16}H_{24}N_2O_6Na$ $(M+Na)^+$: 363.15. Found: m/z 363.13.

5.17. *N*-{[Tris(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyloxy)methyl]methyl}-(4-benzyloxycarbonylamino)-butyramide (28)

Compound **27** (100 mg, 0.294 mmol) and ethyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-galactopyranoside (**20**)[27] (753 mg, 1.17 mmol) were stirred with 4 Å molecular sieves (500 mg) in DCE/Et_2O (20 ml, 1:1) at rt under argon for 2 h. The mixture was cooled to 0 °C, and a solution of NIS (263 mg, 1.17 mmol) and TfOH (0.117 mmol, 10.2 μl) in DCE/Et_2O (10 ml, 1:1) was added. The resultant mixture was stirred at 0 °C under argon for 1 h, upon which it turned deep brown. The mixture was diluted with DCM (50 ml), filtered through Celite, washed with $Na_2S_2O_3$ (1 M, 25 ml), followed by $NaHCO_3$ (0.1 M, 25 ml), and dried with Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by silica gel chromatography (petrol ether/EtOAc 3:1 → 1:1) to yield **28** (311 mg, 51%, R_f 0.23 B) as a colorless solid. $[\alpha]_D$ +39 (c 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.65 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 1.86 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.09 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.52 (d, A of AB, J = 10.2 Hz, 3H, 3× CH_2O-Gal-H_a), 3.72 (m, 3H, 3× H5-Gal), 4.10 (d, $J_{1,2}$ = 8.0 Hz, 3H, 3× H1-Gal), 4.32 (dd, $J_{5,6'}$ = 7.4, $J_{6,6'}$ = 11.2 Hz, 3H, 3× H6′-Gal), 4.35 (d, B of AB, J = 10.1 Hz, 3H, 3× CH_2O-Gal-H_b), 4.51 (dd, $J_{5,6}$ = 6.2, $J_{6,6'}$ = 11.2 Hz, 3H, 3× H6-Gal), 5.02 (m, 2H, CH_2Ph), 5.32 (s, 1H, NH, Cbz), 5.38 (dd, $J_{4,3}$ = 3.3, $J_{2,3}$ = 10.3 Hz, 3× H3-Gal), 5.62 (dd, $J_{1,2}$ = 8.1, $J_{2,3}$ = 10.2 Hz, 3× H2-Gal), 5.75 (s, 1H, NH, Tris), 5.84 (d, $J_{3,4}$ = 3.2 Hz, 3H, 3× H4-Gal), 7.21–8.10 (m, 65H, 1 3× C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 25.2 ($NCH_2CH_2CH_2C{=}O$), 33.8 ($NCH_2CH_2CH_2C{=}O$), 40.1 ($NCH_2CH_2CH_2C{=}O$), 59.4 [$C(CH_2O)_3$], 61.4 (3C, 3× C6-Gal), 66.4 (CH_2Ph), 67.8, 67.9 [6C, $C(CH_2O)_3$, 3× C4-Gal], 69.9 (3C, 3× C2-Gal), 71.0, 71.1 (6C, 3× C3-Gal, 3× C5-Gal), 101.8 (3C, 3× C1-Gal), 127.9–136.7 (78C, 13× C_6H_5), 156.5 [N–(C=O)O], 164.9–172.6 (13C, 13× C=O). Anal. Calcd for $C_{118}H_{102}N_2O_{33}$: C, 68.27; H, 4.95; N, 1.35. Found: C, 67.81; H, 5.08; N, 1.36.

5.18. 4-(Benzyloxycarbonylamino)-*N*-{[tris(β-D-galactopyranosyloxy)methyl]methyl}-butyramide (29)

Compound **28** (241 mg, 0.115 mmol) was dissolved in dry MeOH/dioxane (35 ml, 2.5:1), and sodium metal (65 mg, 2.83 mmol) was added. The resultant mixture was stirred at rt under argon for 6 h, after which it was neutralized with Dowex 50X8 (H^+-form), filtered, and the solvent was removed under reduced pressure. The residue was purified on a Sephadex G-25 column, and, after the removal of water in vacuo, washed with Et_2O/DCM (ca. 2:1) to afford the desired product **29** (81.5 mg, 85%, R_f 0.11 F) as a white powder. $[\alpha]_D$ −0.2 (c 1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 1.78 (m, $NCH_2CH_2CH_2C{=}O$), 2.22 (t, J = 7.1 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.16 (t, J = 6.9 Hz, 2H, $NCH_2CH_2CH_2C{=}O$), 3.45–3.54 (m, 9H, 3× H2-Gal, 3× H3-Gal, 3× H5-Gal), 3.69 (dd, $J_{5,6'}$ = 5.2, $J_{6,6'}$ = 11.3 Hz, 3H, 3× H6′-Gal), 3.76 (dd, $J_{5,6}$ = 6.9, $J_{6,6'}$ = 11.4 *Hz*, 3H, 3× H6-Gal), 3.81 (m, 3H, 3× H4-Gal), 3.93 (d, A of AB, J = 10.2 Hz, 3H, 3× CH_2OGal-H_a), 4.27 (d, $J_{1,2}$ = 7.6 Hz, 3H, 3× H1-Gal), 4.32 (d, B of AB, J = 10.2 Hz, 3H, 3× CH_2O-Gal-H_b), 5.07 (s, 2H, CH_2Ph), 7.29–7.35 (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, MeOD): δ = 27.1 ($NCH_2CH_2CH_2C{=}O$); 35.0 ($NCH_2CH_2CH_2C{=}O$), 41.2 ($NCH_2CH_2CH_2C{=}O$); 62.5 (3C, 3× C6-Gal), 67.4 (CH_2Ph); 69.3 [3C, $C(CH_2O)_3$], 70.4 (3C, 3× C4-Gal), 72.6 (3C, 3× C2-Gal), 74.9 (3C, 3× C3-Gal), 76.7 (3C, 3× C5-Gal), 105.5 (3C, 3× C1-Gal), 127.9–129.5 (6C, C_6H_5), 159.0 [N-(C=O)O], 175.9 (C=O); ESI-MS: Calcd for $C_{34}H_{54}N_2O_{21}Na$ $(M+Na)^+$: 849.31. Found: m/z 849.44.

5.19. 4-Amino-*N*-{[tris(β-D-galactopyranosyloxy)methyl]methyl}-butyramide (30)

Compound **29** (27.2 mg, 32.9 μmol) was dissolved in methanol (2.5 ml), and Pd/C (10% Pd, 25 mg) was added. The resultant mixture was vigorously stirred under a H_2 atmosphere (1 atm) at rt for 48 h. The mixture was then filtered and concentrated in vacuo to yield **30** as a colorless solid (19.9 mg, 87%). $[\alpha]_D$ +3.1 (c 1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 1.89 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 2.32 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.00 (m, 2H, $NCH_2CH_2CH_2C{=}O$), 3.43–3.50 (m, 9H, 3× H2-Gal, 3× H3-Gal, 3× H5-Gal), 3.68 (dd, $J_{5,6'}$ = 5.1, $J_{6,6'}$ = 11.2 Hz, 3H, 3× H6′-Gal), 3.72 (dd, $J_{5,6}$ = 6.9, $J_{6,6'}$ = 11.3 *Hz*, 3H, 3× H6-Gal), 3.77 (m, 3H, 3× H4-Gal), 3.87 (d, A of AB, J = 10.1 Hz, 3H, 3× CH_2OGal-H_a), 4.25 (d, $J_{1,2}$ = 6.8 Hz, 3H, 3× H1-Gal), 4.31 (d, B of AB, J = 10.1 Hz, 3H, 3× CH_2OGal-H_b); ^{13}C NMR (125 MHz, MeOD): δ = 24.5 ($NCH_2CH_2CH_2C{=}O$), 34.7 ($NCH_2CH_2CH_2C{=}O$), 40.3 ($NCH_2CH_2CH_2C{=}O$), 61.3 [$C(CH_2O)_3$], 62.6 (3C, 3× C6-Gal), 69.1 [3C, $C(CH_2O)_3$], 70.8 (3C, 3× C4-Gal), 73.0 (3C, 3× C2-Gal), 75.3 (3C, 3× C3-Gal), 77.1 (3C, 3× C5-Gal), 105.8 (3C, 3× C1-Gal), 174.9 (C=O); ESI-MS: Calcd for $C_{26}H_{49}N_2O_{19}$ $(M+H)^+$: 693.29. Found: m/z 693.39.

5.20. Fluorescent-labeled, Gal-terminated control compound (8)

A stock solution containing compound **30** (12 mg, 17.3 μmol), DIPEA (50 μl, 291 μmol), and 4 Å molecular sieves (50 mg) in dry DMF (1 ml) was stirred at rt under argon for 2 h. The resultant mixture (500 μl) was transferred to a small vial containing Alexa Fluor® 488-NHS (1 mg, 1.55 μmol), 4 Å molecular sieves (25 mg), and a stirring bar. The mixture was then stirred

in the dark at rt under argon for 4 days. The mixture was then diluted with MeOH, filtered, and the solvents were removed in vacuo. The residue was purified on an RP-18 column (H_2O/MeOH gradient 1:0→19:1) to yield **8** (1.8 mg, 96%) as a red solid after final lyophilization from water. ESI-MS: Calcd for $C_{47}H_{59}N_4Na_2O_{29}S_2$ $(M+2Na+H)^+$: 1253.25. Found: *m/z* 1253.59.

5.21. Ligand binding and internalization

5.21.1. Fluorescence microscopy. One day before the experiments, the cells were seeded at a density of 2×10^5 cells/well into 12-well plates containing collagen coated glass coverslips. The cells were washed once with PBS, and then serum-starved for 30 min on ice in 1 ml of DMEM containing 25 mM Hepes. They were then incubated with 500 μl/well of the Alexa Fluor® 488-labeled compounds **6**–**8** (100 μM) in the same medium on ice for 1.5 h in the dark. After the binding step, the cells were washed carefully four times with cold PBS. Then fresh, prewarmed, complete DMEM medium (1 ml/well) was administered, and the cells were incubated for 40 min in an incubator at 37 °C in a humidified CO_2 atmosphere (5%, v/v), leading to the internalization of the receptor-bound compounds into the cells. After the internalization step, the cells were washed twice with PBS and then fixed with 3% paraformaldehyde (PFA) in PBS for 30 min at 4 °C. After fixation, the coverslips were washed abundantly with PBS and mounted upside down, in a Mowiol 4-88 mounting buffer containing *N*-propyl gallate, onto glass slides.

Selective cellular uptake of the Alexa Fluor® 488-labeled compounds was visualized using a Zeiss Axiovert 135 microscope with a 63× planapo objective (numerical aperture = 1.4, oil) with the appropriate filter set (450/490, FT 510, LP 520) equipped with a Zeiss AxioCam MRm CCD camera run by AxioVision 3.1 imaging software.

5.21.2. Flow cytometry. Cells were grown for 24 h in collagen coated (80 μg/ml) 24-well plates at a density of 3×10^5 cells/well or in 96-well plates at 1.5×10^5 cells/well.

The titration experiments were performed in 24-well plates, the cell layers were first washed twice with cold PBS before incubation with compound **7** at concentrations ranging from 0.4 to 12.5 μM (1:2 serial dilutions) in 200 μl of DMEM without FBS for 40 min at 37 °C. Then, the cells were washed twice with cold PBS, detached and stripped from surface-bound compound by incubating them in a mixture containing 0.025% trypsin and 5 mM EDTA in PBS for 10 min on ice. The addi-

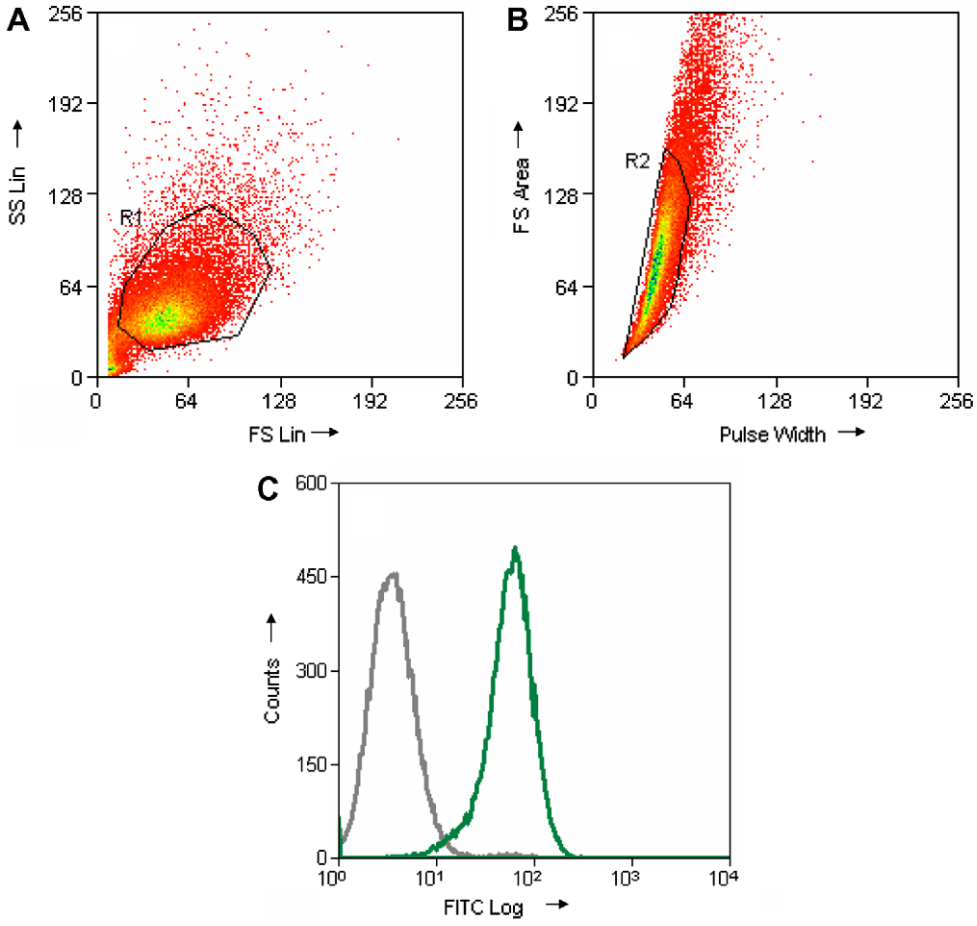


Figure 7. Example of flow cytometric analysis for the uptake of compound **7** into HepG2 cells. All experiments were analyzed in the same way. SK-Hep1 cells, which are less susceptible to clumping, show less debris in dot plot A and sharper peaks in histogram C (data not shown).

tion of complete medium quenched this process. The detached cells were collected and centrifuged at a speed of 1500 rpm for 3 min. Finally, the cells were fixed in 2% PFA in PBS for 15 min on ice followed by an aldehyde-quenching step with 100 mM lysine in PBS for 10 min. The fixed cells were then washed once with FACS buffer (PBS containing 0.5% BSA and 0.1% NaN_3) and resuspended in 200 μl of the same buffer for measuring.

The competitive uptake experiments were performed with cells grown in 96-well plates. Twenty microliters of asialofetuin dilutions at concentrations ranging from 0.6 to 200 μM or Gal*N*Ac at 0.6–200 mM was added directly to the cells, immediately followed by the addition of 20 μl of compound **7** or **8** diluted to 20 μM in DMEM without FBS and then incubated for 60 min at 37 °C in the incubator. The cells were washed, detached, and fixed as described above.

Analyses were performed on a CyAn ADP flow cytometer with Summit 4.1 software (Dako Cytomation). An example for the analysis of HepG2 cells is given in Figure 7. The forward and side scatter gate R1 was set to count 30,000 intact cells of each sample (Fig. 7, dot plot A). In gate R2, the cells counted in gate R1 were discriminated for doublets (Fig. 7, dot plot B). Histogram C depicts an overlay of the log of fluorescence intensity at 488 nm of untreated (gray) and treated HepG2 cells (green) from gate R2. The uptake of a compound into cells was evaluated by comparing the shift in median intensity of fluorescence (MFI) between untreated cells (background fluorescence) and treated cells.

Further analysis and IC_{50} calculations were done with GraphPad Prism 4 software.

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