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# Design, synthesis and evaluation of monovalent ligands for the asialoglycoprotein receptor (ASGP-R)

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ABSTRACT

A series of novel aryl-substituted triazolyl D-galactosamine derivatives was synthesized as ligands for the carbohydrate recognition domain of the major subunit H1 (H1-CRD) of the human asialoglycoprotein receptor (ASGP-R). The compounds were biologically evaluated with a newly developed competitive binding assay, surface plasmon resonance and by a competitive NMR binding experiment. With compound **1b**, a new ligand with a twofold improved affinity to the best so far known D-Gal*N*Ac was identified. This small, drug-like ligand can be used as targeting device for drug delivery to hepatocytes.



## 1. Introduction

The asialoglycoprotein receptor (ASGP-R) is a C-type,[1] i.e. a $Ca^{2+}$-dependent lectin and is expressed exclusively by parenchymal hepatocytes, which contain 100,000–500,000 binding sites per cell. These receptors are randomly distributed over the sinusoidal plasma membrane facing the capillaries. Their main function is to maintain serum glycoprotein homeostasis by recognition, binding, and endocytosis of asialoglycoproteins (ASGPs). After internalization via receptor-mediated endocytosis, the ASGPs dissociate in the acidic environment of the endosomes and are finally degraded, while the receptor is recycled back to the cell surface.[2–5]

The human ASGP-R consists of two homologous subunits, designated H1 and H2, which form a non-covalent heterooligomeric complex by α-helical coiled coil domains with an estimated ratio of 2–5:1, respectively.[6,7] Both subunits are single-spanning membrane proteins with a calcium-dependent D-Gal/D-Gal*N*Ac recognition domain.[8] Whereas the recognition of the carbohydrate moiety is mediated via the H1-subunit, the H2-subunit accounts for the functional configuration of the native receptor.[9–12] Recently, the X-ray crystal structure of the carbohydrate recognition domain (CRD) of the major H1-subunit was solved.[13]

Beyond the specificity for terminal D-galactose and *N*-acetyl-D-galactosamine residues, binding to the ASGP receptor strongly depends on the valency of the ligand. Whereas the affinity of a single D-galactose residue is only in the millimolar range, bi-, tri- and tetraantennary desialylated glycoproteins bind with dissociation constants of $10^{-6}$, $5 \times 10^{-9}$, and $10^{-9}$ M, respectively.[14,15] Furthermore, Lee and co-workers demonstrated that the ASGP-R exhibits an approximately 10–50-fold higher affinity for D-Gal*N*Ac versus D-Gal monosaccharides[16] and for terminal D-Gal*N*Ac versus D-Gal cluster glycosides,[17] respectively. As a result, research efforts for the design of high affinity ligands were focused on oligovalent ligands, containing primarily D-Gal*N*Ac but also D-Gal or D-Lac as terminal recognition elements. Their use as homing devices in liver-specific drug[18,19] and gene delivery[20–24] is well documented.

By contrast, only moderate research efforts were directed towards the discovery of potent monovalent ligands. Lee et al.[25] showed that the replacement of the acetamido group in D-Gal*N*Ac by a propanamido or trifluoroacetamido group did not lead to an alteration of the inhibitory effect regarding $^{125}$I-asialo-orosomucoid binding to rat hepatocytes. However, with the bulkier benzamido or phthalimido groups an up to 10-fold drop in affinity was observed, indicating the impact of sterical factors on binding affinity. Later on, Weis and co-workers[26,27] provided a sophisticated model based on the mannose-binding protein to explain

*Abbreviations:* ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); AIBN, azo-bis-isobutyronitrile; ASGP, asialoglycoprotein; CRD, carbohydrate recognition domain; DCM, dichloromethane; D-Gal, D-galactose; D-Gal*N*Ac, *N*-acetyl-D-galactosamine; D-Lac, D-lactose; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; $K_D$, dissociation constant; NMR, nuclear magnetic resonance; PAA, polyacrylamide; SPR, surface plasmon resonance; Tf, trifluormethanesulfonyl.

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the preferential binding of D-Gal*N*Ac compared with D-Gal as well as for the selectivity of different *N*-acyl derivatives.

For our investigation, we designed a directed library of easy accessible and metabolically stable D-galactosamine derivatives for mono- or oligovalent applications, for example, for the incorporation into trivalent drug carriers previously developed.[28] Furthermore, for the efficient evaluation of binding affinities the hitherto used radio-labeled competitive binding assay[29–31] was replaced by a non-radioactive, polymer-based assay format. Finally, surface plasmon resonance and NMR experiments allowed an independent validation of the binding affinities.

## 2. Results and discussion

### 2.1. Design of ASGP-R H1 ligands

Docking D-Gal*N*Ac to the crystal structure of the H1-CRD (Fig. 1A and B, PDB code 1DV8)[13] clearly reveals the pharmacophoric groups involved in this carbohydrate–lectin interaction. The 3- and 4-hydroxyl groups of D-Gal*N*Ac coordinate to the $Ca^{2+}$ ion, requiring their equatorial and axial orientation (Fig. 1B).[32] Due to steric reasons, methylation of these hydroxyls leads to a substantial loss in affinity.[25] The hydrophobic patch formed by the circular arrangement of ring C–H bonds on the α-face of D-Gal*N*Ac establishes a lipophilic contact with the indole side chain of Trp243 (Fig. 1A). The pharmacophoric requirements are summarized in Figure 1C.

A closer look at the protein surface in the area of the 2-*N*-acetyl group reveals a dumbbell-shaped cavity (Fig. 1D) that on the one hand quickly leads to a steric clash with bulky substituents,[25] but on the other hand can host a wide variety of linear substituents. Finally, the β-anomeric as well as the 6-OH point into the solvent and could therefore serve as attachment points for conjugation to oligovalent carriers, fluorescent labels, etc.

Based on these evidences, a directed library of small, drug-like D-Gal*N*Ac derivatives was designed where (i) the anomeric OH was removed, since it could act as a metabolic 'soft-spot'[33] and (ii) the 2-acetamido group was replaced by a 4-substituted 1,2,3-triazole moiety. Docking of the test compound **1a** to the H1-CRD revealed a perfect accommodation of the triazole substituent by the dumbbell-shaped hydrophobic binding pocket (Fig. 1D).

### 2.2. Synthesis of triazole derivatives 1a–f, h

In anti-substituted triazoles as **1a**, the substituent in the 4-position of the heterocycle perfectly points into the dumbbell-shaped binding pocket. Anti-substituted triazoles are obtained highly regioselectively by a copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition.[34,35] For the synthesis of the azido component **7** used in the cycloaddition reaction (Scheme 1), peracetylated D-Gal*N*Ac (**2**) was used as starting material. Its treatment with $TiCl_4$ in DCM yielded glycosyl chloride **3** in 89%. Reductive dehalogenation using $Bu_3SnH$ and AIBN in refluxing toluene gave **4** in quantitative yield. Deacetylation (→**5**), followed by amine-azide exchange[36]

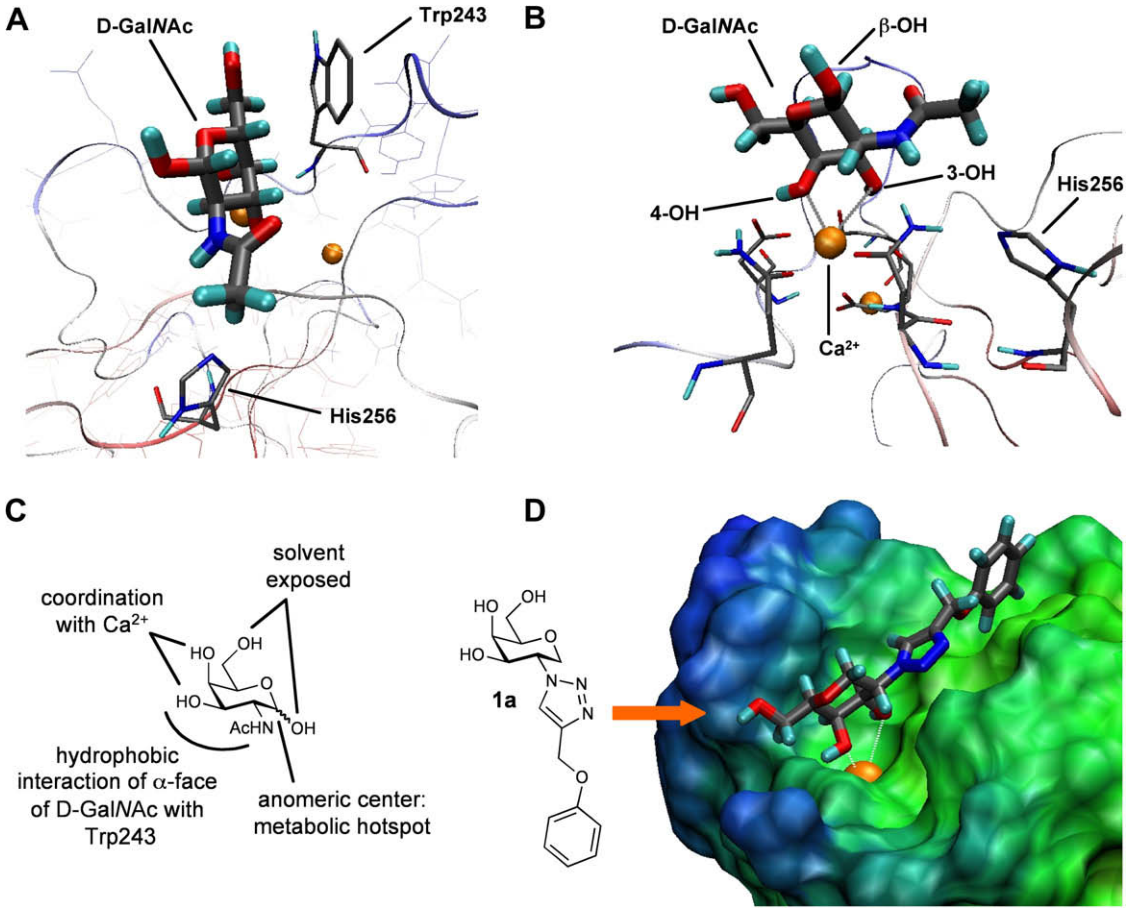
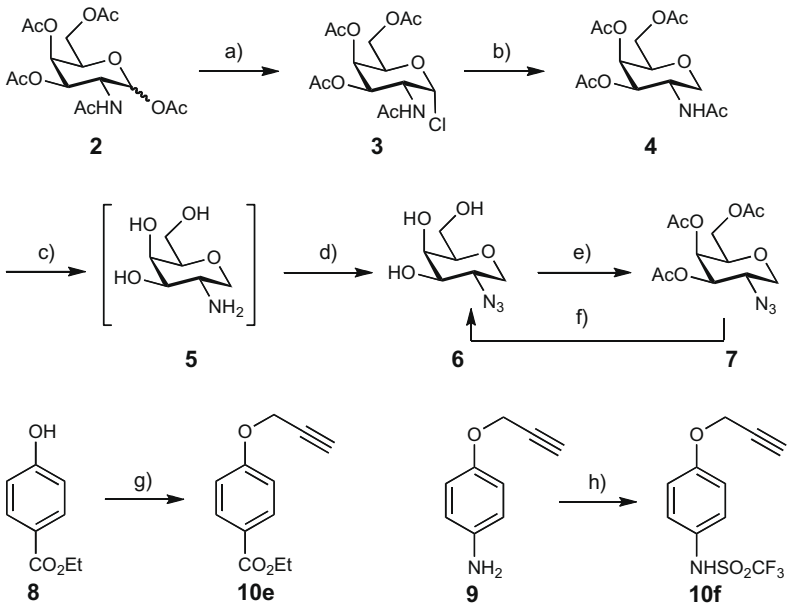



**Figure 1.** (A) Manual docking (MacYeti 7.05) of β-D-Gal*N*Ac into the H1-CRD of the ASGP-R (PDB code 1DV8).[13] Illustration of the hydrophobic interactions between Trp243 and the α-face of D-Gal*N*Ac and the *N*-acetate group with His256;[27] (B) coordination of the equatorial 3- and axial 4-hydroxyl group of D-Gal*N*Ac with $Ca^{2+}$; (C) summary of the important interactions of D-Gal*N*Ac with the binding site of the H1-CRD; (D) a model of the H1-CRD interacting with the D-Gal*N*Ac derivative **1a**, with the 3- and 4-OH groups coordinating $Ca^{2+}$ (orange) and the triazole substituent well accommodated by a dumbbell-shaped hydrophobic binding pocket.

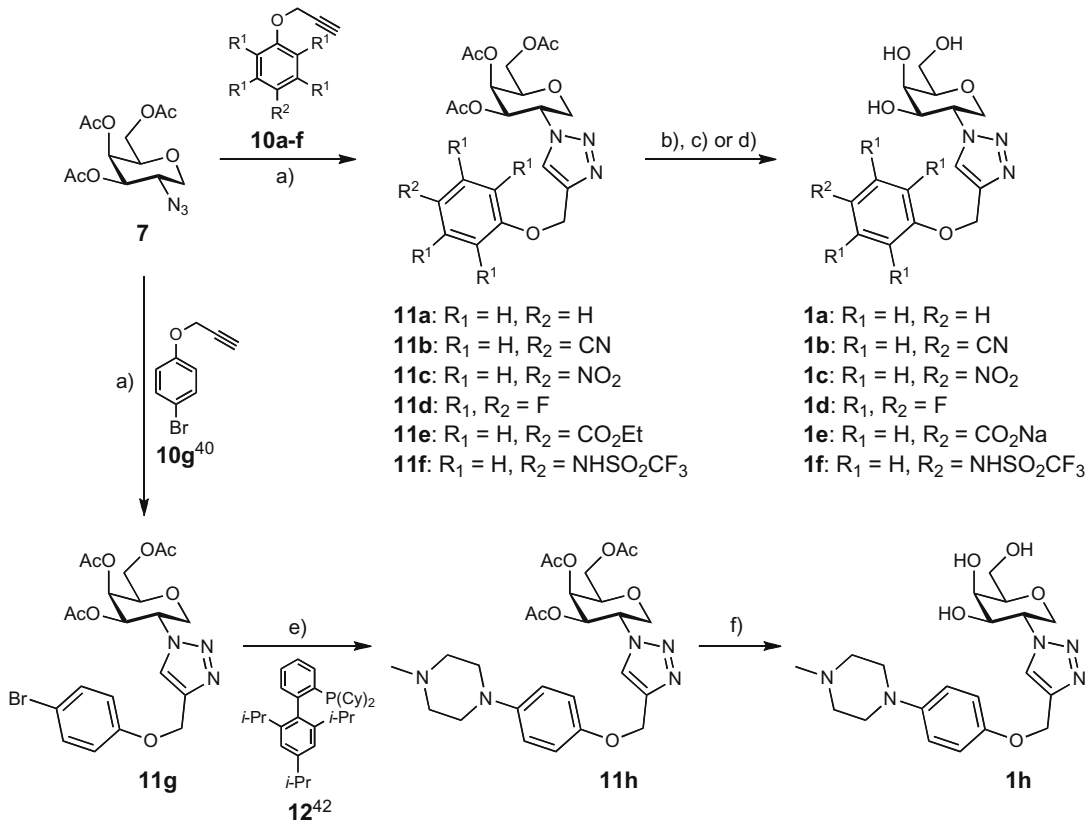
(→**6**) and subsequent re-acetylation gave **7** in 76% yield over three steps. For affinity testing, a small sample of **7** was deprotected under standard Zemplén conditions to yield **6** quantitatively.

Whereas acetylene **10a** (Scheme 2) is commercially available, **10b–d** and **10g** were easily synthesized following known procedures.[37–40] **10e** was obtained quantitatively by propargylation of 4-hydroxybenzoic acid (**8**) and **10f** in 69% by sulfonylation of **9**[37] with triflic anhydride (Scheme 1).

Cu(I)-catalyzed click conditions,[34,35] i.e. $CuSO_4$ and sodium ascorbate as reducing agent to obtain the catalytically active




**Scheme 1.** Synthesis of building blocks. Reagents and conditions: (a) $TiCl_4$, DCM, rt, 3 d, 89%; (b) $Bu_3SnH$, AIBN, PhMe, reflux, 1 h 20 min, quant.; (c) KOH, 18-crown-6, dioxane/$H_2O$, reflux, 6.5 h; (d) $TfN_3$, $NaHCO_3$, $CuSO_4{\cdot}5H_2O$, $H_2O$/PhMe/MeOH, rt, 24 h; (e) $Ac_2O$, pyridine, rt, 24 h, 76% (three steps); (f) NaOMe/MeOH, rt, 3 h, quant.; (g) propargyl bromide, $K_2CO_3$, acetone, reflux, 5 h, 73%; (h) $Tf_2O$, $Et_3N$, DCM, 0 °C→rt, 24 h, 69%.




**Scheme 2.** Synthesis of triazoles. Reagents and conditions: (a) CuCl, $Et_3N$, DCM, rt, 24 h, **10a** (→**11a**, 93%), **10b**[38] (→**11b**, 98%), **10c**[37] (→**11c**, 95%), **10d**[39] (→**11d**, 73%), **10e** (→**11e**, 82%), **10f** (→**11f**, 41%), or **10g**[40] (→**11g**, 84%); (b) NaOMe/MeOH, rt, 3 h (**1a**, 45%; **1c**, 33%; **1d**, 68%; **1f**, 73%); (c) $H_2O$/MeOH/$Et_3N$ (5:5:1), rt, 24 h (**1b**, 81%); (d) NaOH, $H_2O$/MeOH, rt, 24 h (**1e**, 74%); (e) *N*-methylpiperazine, $Pd_2(dba)_3$, $Cs_2CO_3$, PhMe, 80 °C, 24 h; (f) NaOMe/MeOH, rt, 24 h (44%, two steps).

Cu(I), led only to low yields of anti-substituted triazoles and in some cases even failed completely. However, with an alternative procedure, involving CuCl as the direct source of Cu(I) and triethylamine as base, the cycloaddition reactions of azide **7** with the phenyl propargyl ethers **10a**–**f** yielded the protected *anti*-substituted triazoles **11a**–**f** in 41–98% yield and after deprotection the test compounds **1a**–**f** (Scheme 2). To further explore the dumbbell-shaped binding site and to improve solubility of the test compound, a piperazinylphenyl derivative was synthesized. Click chemistry (→**11g**) and Pd-catalyzed Buchwald–Hartwig coupling using the biaryl monophosphine ligand **12** (→**11h**)[41] followed by deacetylation by Zemplén conditions yielded **1h**.

### 2.3. Expression of H1-CRD

A truncated form of the H1-subunit of the human hepatic ASGP-R consisting of the whole CRD domain (amino acid residues 147–291) was recombinantly expressed in *Escherichia coli* and purified to homogeneity by affinity chromatography.[43] The monomeric and dimeric forms were subsequently purified by ion exchange chromatography. The purified proteins were analyzed by SDS–PAGE and immuno-blot (Fig. 2).

### 2.4. Competitive binding assay

For the characterization of the H1-CRD ligands, a cell-free, competitive binding assay based on a polyacrylamide (PAA) glycoconjugate was established analog to binding assays reported for selectin antagonists.[44,45] For this assay, a microtiter plate coated with the H1-CRD is incubated with biotinylated D-Gal*N*Ac-PAA conjugated to streptavidin-horseradish peroxidase. Then, after incubation with the analyte at various concentrations and washing with HBS-buffer, the remaining PAA glycoconjugate is quantified by a colorimetric reaction using the enzyme's substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS). PAAs containing β-D-Glc or sLe$^a$ are not recognized, whereas H1-CRD efficiently bound β-D-Gal*N*Ac-PAA and to a lesser extent β-D-Gal-PAA (Fig. 3A). Since the interaction is $Ca^{2+}$-dependent, D-Gal*N*Ac-polymer binding is completely inhibited in the presence of 1 mM EDTA in the assay buffer (Fig. 3B).

For a further validation of the binding assay, the $IC_{50}$'s of a number of monosaccharides were compared with $K_D$ values derived from surface plasmon resonance (SPR, Biacore) measurements, were the H1-CRD was immobilized on a CM5 chip by amine coupling, and $IC_{50}$'s reported in literature.[16]

Interestingly, consistently negative sensorgrams in the surface plasmon resonance experiments, that is, a net decrease in resonance units, were obtained with the monosaccharides reported in Table 1. When fitted to a binding isotherm, these negative sensorgrams appear to clearly result from specific receptor–ligand interactions. Numerous factors such as the buffer capacity, the ion-strength of the buffer and matrix type of the sensor chip could be excluded as direct cause of the negative sensorgrams. A possible explanation is a ligand-induced conformational change of the

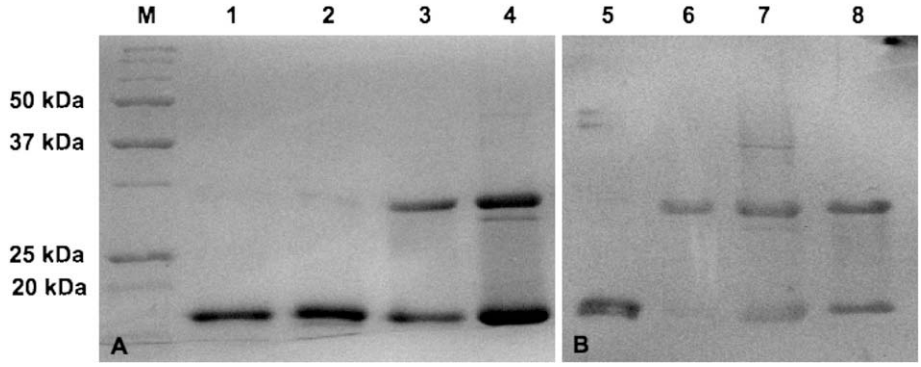



**Figure 2.** SDS–PAGE and immuno-blot analysis. (A) Monomeric and dimeric forms of H1-CRD were separated on a non-reducing 15% SDS–PAGE and visualized by coomassie staining. M: molecular weight marker, 1 and 2: H1-CRD monomer, 3 and 4: H1-CRD monomer and dimer; (B) Immuno-blot analysis was performed with IgY polyclonal anti-H1-CRD antibodies and alkaline phosphatase coupled rabbit anti-chicken IgG. 5: H1-CRD monomer, 6: H1-CRD dimer, 7 and 8: H1-CRD monomer and dimer.

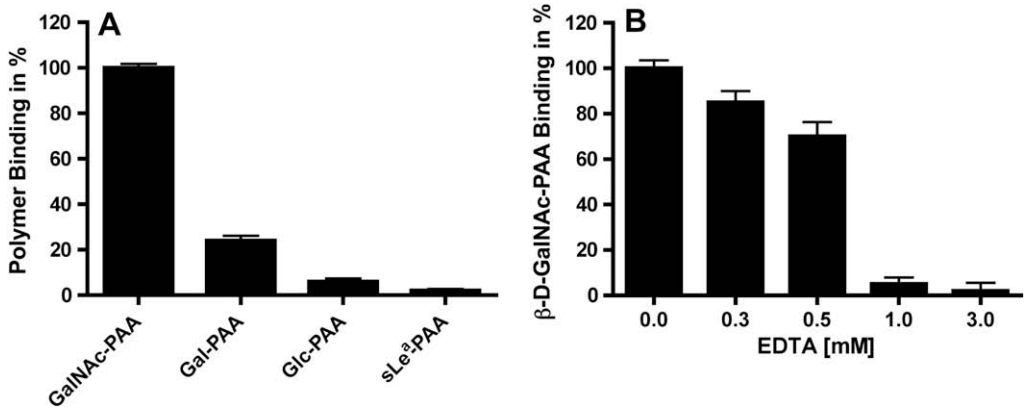



**Figure 3.** (A) Specificity of the binding assay. Comparison of H1-CRD binding of β-D-Gal*N*Ac-PAA and β-D-Gal-PAA and the negative controls β-D-Glc-PAA and sLe$^a$-PAA; (B) effect of EDTA on β-D-Gal*N*Ac-PAA binding to H1-CRD.

**Table 1**
Affinity data obtained by the competitive binding assay ($IC_{50}$) and surface plasmon resonance ($K_D$)

| Monosaccharides | Competitive binding assay[a] (human H1) | | Surface plasmon resonance (human H1) | | Literature data[16] (rabbit ASGP-R) | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ ± SD [μM] | $rIC_{50}$ | $K_D$ ± SD [μM] | $rK_D$ | $IC_{50}$ [μM] | $rIC_{50}$ |
| D-Gal*N*Ac | 113 ± 13 | 1 | 150 ± 0.9 | 1 | 90 | 1 |
| D-Gal | 1049 ± 101 | 9.3 | 1460 ± 10 | 9.7 | 1700 | 18.9 |
| β-D-GalOMe | 1828 ± 169 | 16 | 2200 ± 40 | 14.7 | 1000 | 11.1 |
| α-D-GalOMe | 2831 ± 140 | 25 | 2760 ± 60 | 18 | 1600 | 17.8 |
| D-Glc | >30,000 | n.d. | >10,000 | n.d. | 60,000 | 667 |

$IC_{50}$ defines the molar concentration of the test compound that reduces the maximal specific binding of D-Gal*N*Ac-PAA to H1-CRD by 50%. The relative $IC_{50}$ ($rIC_{50}$) is the ratio of the $IC_{50}$ of the test compound to the $IC_{50}$ of the reference compound D-Gal*N*Ac. SD: standard deviation. Literature data describe the inhibition of $^{125}$I-asialoorosomucoid binding to isolated rabbit hepatic lectin.[16]

[a] Compounds tested in triplicates, each at six different concentrations. The coefficient of variation, which is defined as the standard deviation divided by the mean, was 13.8% for unspecific binding and 4.8% for specific binding. These results demonstrate the low variability of the assay, making it suitable for the characterization of H1-CRD ligands.

immobilized receptor leading to a decrease of its hydrodynamic radius and, as a consequence, yielding a negative refraction index.[46] Since the negative refraction index correlates with the analyte concentration, we mirrored the negative sensorgrams by multiplication of each data point with −1. The $K_D$s obtained correlate nicely with affinity data derived from the competitive binding assay and from the literature values (Table 1).

All three assay formats revealed a 10–20-fold higher affinity for D-Gal*N*Ac compared to D-Gal. Interestingly, methyl β-D-galactoside binds with slightly higher affinity than the corresponding α-anomer, although according to docking studies (see Fig. 1B) both aglycons point to the solvent. Furthermore, D-Glc-binding was beyond the detection limit, supporting the importance of the coordination of the 3- and 4-hydroxyls to $Ca^{2+}$. Overall, the three assay formats gave consistently comparable results, confirming the reliability of our new competitive binding assay.

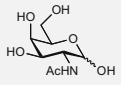
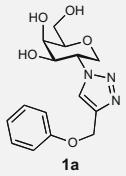
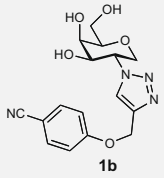
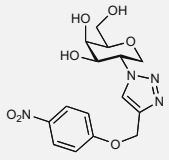
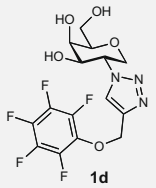
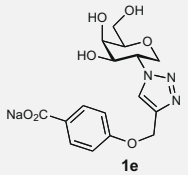
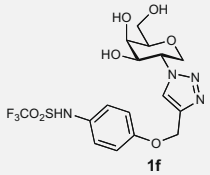
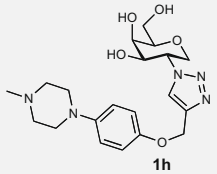
The affinities of the triazoles are summarized in Table 2 and Figure 4. Compounds that were not soluble in HBS-buffer were dissolved in DMSO prior to dilution with buffer. The final DMSO concentration in the diluted samples did not exceed 5% and was well tolerated in the assay (data not shown).

Not unexpectedly, the azide scaffold **6** (Table 2, entry 2) exhibited an approximately 14-fold decrease in affinity compared to D-Gal*N*Ac (entry 1), because the proposed hydrophobic contact of the *N*-acetate group with His256[27] is no longer present. On the other hand, compound **1a** (entry 3), bearing a phenyloxymethyl-substituted triazole, had an affinity close to the one of D-Gal*N*Ac ($rIC_{50}$ 1.1), indicating that the rather bulky substituent in the 2-position is tolerated. Only the *p*-cyano derivative **1b** (entry 4) and the *p*-nitro-derivative **1c** (entry 5) showed clearly better affinities than D-Gal*N*Ac. Despite its bulkiness, the *N*-methylpiperazine derivative **1h** exhibited a slightly improved $rIC_{50}$ compared to D-Gal*N*Ac. This result further supports our hypothesis that compounds extended at the 2-position of the amino pyranose ring can interact with the dumbbell-shaped binding pocket (see Fig. 1D).

## 2.5. Competitive NMR binding experiments

For an independent assessment of the binding affinities, competitive binding experiments by NMR[47,48] were performed with D-Gal*N*Ac as a reference compound and **1b** as competitor. In a first step, binding of D-Gal*N*Ac to the H1-CRD was demonstrated by the large difference in transverse relaxation times in the absence and presence of the lectin (Fig. 5A).

**Table 2**
Determination of the relative $IC_{50}$ ($rIC_{50}$) values for the triazolyl D-galactosamine derivatives

| Entry | Compound | $rIC_{50}$ ± SD |
|---|---|---|
| 1 |  | 1.0 |
| 2 |  **6** | 13.7 ± 4.3 |
| 3 |  **1a** | 1.1 ± 0.2 |
| 4 |  **1b** | 0.5 ± 0.1 |
| 5 |  **1c** | 0.8 ± 0.2 |
| 6 |  **1d** | 1.4 ± 0.3 |
| 7 |  **1e** | 8.7 |
| 8 |  **1f** | —[a] |
| 9 |  **1h** | 0.9 ± 0.1 |

SD: standard deviation.

[a] Compound not soluble in the 10% DMSO/buffer stock solution used in the assay.

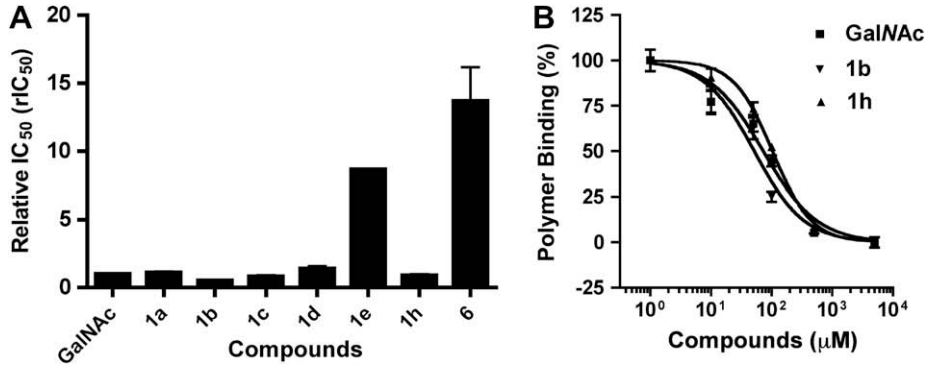



**Figure 4.** (A) Relative $IC_{50}$s derived from the competitive solid-phase binding assay; (B) Plot of D-Gal*N*Ac-PAA inhibition versus concentration. Inhibition of D-Gal*N*Ac-PAA binding to H1-CRD by Gal*N*Ac and derivatives. Microtiter plates were coated with a 3 μg/mL solution of monomeric H1-CRD. After washing steps a serial dilution of the test compound solution and 0.5 μg/mL of streptavidin-peroxidase coupled D-Gal*N*Ac-PAA were added to the protein. After 2 h incubation the plates were washed and the colorimetric reaction was developed and quantified as described in experimental section. (■) Gal*N*Ac, (▼) **1b**, (▲) **1h**.

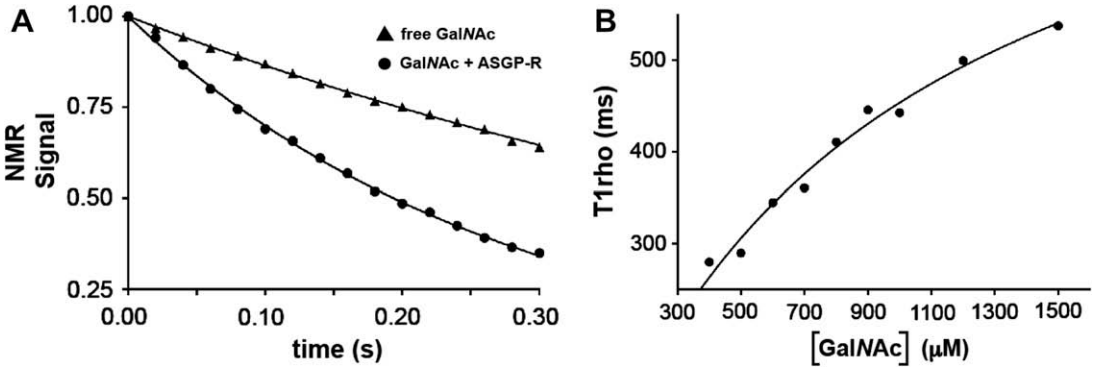



**Figure 5.** (A) Transverse relaxation of D-Gal*N*Ac, either in the presence (circles) or in the absence (triangles) of ASGP-R H1-CRD. T1rho experiments;[47] (B) Titration of ASGP-R H1-CRD with D-Gal*N*Ac and the corresponding T1rho time constants. The leftmost point, at 400 μM D-Gal*N*Ac, is the T1rho time constant determined for the data in Figure 5B. Additional points correspond to the identical analysis with increasing D-Gal*N*Ac concentrations.

For the quantitative evaluation of the relative affinity of **1b** relative to D-Gal*N*Ac, a titration curve documenting the changes in the transverse relaxation times of D-Gal*N*Ac was required. The T1rho time constants were therefore measured for different concentrations of D-Gal*N*Ac and fit to a one-site binding model (Fig. 5B). The relative affinities were then determined in a two-step process. In a first step, a NMR sample with H1-CRD and the concentration of D-Gal*N*Ac corresponding to the initial point of the titration curve (400 μM) was investigated, indicating a high degree of reproducibility according to the nearly identical T1rho time constants for the first and second sample (282 ± 2 ms and 280 ± 3 ms, respectively). The second step consisted of mixing a small amount of the competitor with the second sample (H1-CRD, 400 μM of D-Gal*N*Ac) and measuring the resulting T1rho time constant, from which the apparent concentration of D-Gal*N*Ac was determined by means of the titration curve. When 200 μM of **1b** were added, the T1rho time constant increased to 401 ± 2 ms. Interpolating this value on the titration curve in Figure 5B indicates an apparent concentration of D-Gal*N*Ac of 785 μM. By subtracting the actual concentration for D-Gal*N*Ac from its apparent concentration and dividing through the concentration of **1b** a relative affinity of 1.9 with respect to D-Gal*N*Ac was obtained, a result almost identical to that received by the competitive solid-phase assay.

## 3. Conclusion

Based on docking studies of D-Gal*N*Ac to the crystal structure of the H1-CRD of the ASGP-R, a directed library of aryl-substituted triazole derivatives was synthesized and biologically evaluated. For that purpose, a competitive binding assay allowing the determination of $Ca^{2+}$-dependent binding of potential ligands to the H1-CRD was developed. The reliability of this sensitive, rapid and simple assay was confirmed by a comparison with $IC_{50}$'s reported in literature and $K_D$ values derived from SPR measurements.

The presented triazolyl galactosamine derivatives which are synthetically easily accessible and hydrolytically stable have drug-like properties. Thus, **1b** may be used instead of D-Gal*N*Ac for oligovalent drug-carriers directed to the ASGP-R, for example, with the scaffold we recently published.[28] Eventually, improved affinity may be realized by further modifications of the substituents of the triazole ring. These follow-up experiments are currently being performed.

It remains to be seen whether the new ligands selectively bind to the H1-CRD of the ASGP-R or bind equally well to the three other D-Gal/D-Gal*N*Ac-receptors of the C-type lectin family,[49] the Kupffer cell receptor, the macrophage galactose lectin, and the scavenger receptor C-type lectin (SRCL).[50–53]

## 4. Experimental

### 4.1. General methods

NMR spectra were recorded on a Brucker Avance DRX-500 (500 MHz) spectrometer. Assignment of $^1H$ and $^{13}C$ NMR spectra was achieved using 2D methods (COSY, HSQC, TOCSY). Chemical shifts are expressed in ppm using residual $CHCl_3$, $CHD_2OD$ and

HDO as references. NMR solvents were purchased from Sigma–Aldrich ($CDCl_3$), Armar Chemicals (MeOH-$d_4$, $D_2O$) and Cambridge Isotope Laboratories (Tris-$d_{11}$, DMSO-$d_6$). Optical rotations were measured using a Perkin–Elmer Polarimeter Model 341. The LC/HR-MS analysis were carried out using a Agilent 1100 LC equipped with a photodiode array detector and a Micromass QTOF I. Reactions were monitored by TLC using glass plates coated with Silica Gel 60 $F_{254}$ (Merck). 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% $H_2SO_4$). 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). 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) was dried by filtration over $Al_2O_3$ (Fluka, type 5016 A basic). Dioxane and toluene were dried by refluxing with sodium and benzophenone. Molecular sieves (4 Å) were activated in vacuo at 500 °C for 2 h immediately before use.

The vector pET3b encoding the amino acid residues 147–291 of H1-subunit, was kindly provided by M. Spiess (University of Basel, Switzerland). *E. coli* strain AD494 (DE3) was purchased from Novagen (Lucerne, Switzerland). Bacto-Yeast extract, Bacto-Agar and Bacto-Tryptone for LB (Luria-Bertani) and TB (Terrific Broth) culture media were purchased from Becton Dickinson (Allschwil, Switzerland). IPTG (isopropyl β-D-thiogalactopyranoside) was from AppliChem. Sepharose-4B column material, kanamycin, carbenicillin and protein-standard BSA solution were obtained from Sigma (Buchs, Switzerland). The Shodex IEC-DEAE column was from Brechtbühler (Schlieren, Switzerland). Biotinylated polyacrylamide polymers (D-Gal*N*Ac-, D-Gal-PAA) were obtained from Lectinity (Moscow, Russia). HEPES [4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid], oxalic acid, $CaCl_2$ and methyl β-D-glucopyranoside were from Fluka (Buchs, Switzerland). Methyl α- and β-D-galactopyranoside and bovine serum albumin (BSA) were from Sigma (Buchs, Switzerland). Lactose, *N*-acetyl-D-galactosamine and D-galactosamine were obtained from Pfanstiehl Laboratories (Ohio, USA). Streptavidin-peroxidase conjugate was from Roche Applied Science (Switzerland). Fetal bovine serum (FBS) was from Invitrogen. The peroxidase substrate kit ABTS [2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid)] was obtained from BioRad (California, USA) and MaxiSorp 96-well microtiter plates were from Nunc (Roskilde, Denmark). Anti-H1-CRD polyclonal antibodies were produced in chicken and purified from their eggs after PEG-precipitation[56] by affinity chromatography on a agarose-immobilized H1-CRD column. Surface plasmon resonance experiments were performed on a Biacore 3000 machine using CM5 chips (Biacore AB, Uppsala, Sweden).

#### 4.1.1. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl chloride (3)

Titanium tetrachloride (185 μL, 1.69 mmol) was added to a suspension of **2** (500 mg, 1.28 mmol) in dry DCM (5 mL). The mixture was stirred at rt under argon for 3 d. The solvent was removed in vacuo, and the residue was purified by flash chromatography on silica gel (petrol ether/EtOAc 2:3→1:4), yielding **3** (419 mg, 89%). The analytical data were identical to those found in the literature.[54]

#### 4.1.2. 2-Acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol (4)

A solution of **3** (2.24 g, 6.12 mmol) in dry toluene (33 mL) was degassed in an ultrasound bath under a steady flow of argon for 30 min. Tributyltin hydride (2.14 g, 7.35 mmol) and AIBN (ca. 25 mg) were added and the mixture was refluxed under argon for 80 min. The solvent was removed in vacuo, and the residue was purified by flash chromatography on silica gel (toluene/EtOAc (1:4→1:9→0:1), yielding **4** (2.03 g, quant).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.93 (s, 3H, NHAc), 2.02, 2.04, 2.14 (3s, 9H, 3 OAc), 3.15 (t, $J$ = 11.1 Hz, 1H, H-1ax), 3.76 (m, 1H, H-5), 4.06 (m, 2H, H-6), 4.18 (dd, $J$ = 5.2, 11.3 Hz, 1H, H-1eq), 4.40 (m, 1H, H-2), 4.92 (dd, $J$ = 3.3, 11.2 Hz, 1H, H-3), 5.35 (m, 1H, H-4), 5.69 (d, $J$ = 8.0 Hz, 1H, NH); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.6, 20.7, 20.8 (3 OCO*C*H$_3$), 23.2 (NHCO*C*H$_3$), 46.5 (C-2), 62.2 (C-6), 67.1 (C-4), 68.6 (C-1), 71.5 (C-3), 75.0 (C-5), 170.25, 170.3, 170.5, 171.4 (4 CO); Anal. Calcd for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39; N, 4.23. Found: C, 51.07; H, 6.49; N, 4.12.

#### 4.1.3. 2-Amino-1,5-anhydro-D-galactitol (5)

To a solution of **4** (3.17 g, 9.58 mmol) in dioxane (8.5 mL) were subsequently added water (6.5 mL), 18-crown-6 (21.1 mg) and potassium hydroxide (840 mg, 15.0 mmol). The reaction mixture was refluxed for 5 h. Another 5 equiv (2.10 g) of potassium hydroxide was then added, and the mixture was refluxed for another 1.5 h. The solvent was removed in vacuo and the crude product **5** (3.91 g) was used without further purification in the next step.

#### 4.1.4. 3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-azido-2-deoxy-D-galactitol (7)

*Triflyl azide stock solution preparation*: Sodium azide (3.49 g, 53.7 mmol) was dissolved in water (8.7 mL). Toluene was added, and the mixture was cooled to 0 °C with stirring. Then triflic anhydride (5.8 mL, 34.4 mmol) was added dropwise. The biphasic reaction mixture was stirred vigorously at 0 °C for 1 h and at 10 °C for another 2 h. The reaction mixture was neutralized with satd aq $NaHCO_3$. The phases were separated, and the aqueous phase extracted with toluene (2 × 8 mL). The organic layers were combined to give the triflyl azide stock solution.

*Amine-azide exchange*: Compound **5** (1.56 g, 9.56 mmol), $NaHCO_3$ (319 mg, 38.0 mmol) and $CuSO_4 \cdot H_2O$ (95.0 mg, 380 μmol) were dissolved in water (6.4 mL). The triflyl azide stock solution (21 mL, 9.56 mmol) was added and the biphasic reaction mixture was made homogenous by the dropwise addition of MeOH. The mixture was stirred at rt overnight during that time the color of the mixture turned from blue to green. The solvent was removed in vacuo and the residue containing **6** (1.81 g) was taken up in dry pyridine (36 mL, 447 mmol), and acetic anhydride (8.2 mL, 86.2 mmol) was added. The reaction mixture was stirred at rt under argon overnight. The solvent was removed in vacuo and the crude product was purified by flash chromatography (petrol ether/EtOAc 20:1→9:1) to give **7** (2.31 g, 76%).

$[\alpha]_D$ +0.04 ($c$ 1.00, $CHCl_3$); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 2.09, 2.16, 2.22 (3s, 9H, 3 OAc), 3.24 (t, $J$ = 11.3 Hz, 1H, H-1ax), 3.79 (dt, $J$ = 0.9, 6.5 Hz, 1H, H-5), 3.93 (m, 1H, H-2), 4.08 (d, $J$ = 6.5 Hz, 2H, H-6), 4.12 (dd, $J$ = 5.4, 11.6 Hz, 1H, H-1eq), 4.91 (dd, $J$ = 3.3, 10.4 Hz, 1H, H-3), 5.40 (d, $J$ = 0.9 Hz, 1H, H-4); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.6, 20.8, 22.1 (3 CO*C*H$_3$), 55.9 (C-2), 61.9 (C-6), 67.1 (C-4), 68.1 (C-1), 73.4 (C-3), 74.9 (C-5); Anal. Calcd for $C_{12}H_{17}N_3O_7$: C, 45.72; H, 5.43; N, 13.33. Found: C, 45.90; H, 5.44; N, 13.18.

#### 4.1.5. 1,5-Anhydro-2-azido-2-deoxy-D-galactitol (6)

Compound **7** (20.0 mg, 63.4 μmol) was dissolved in MeOH (2 mL) and sodium metal (10 mg) was added. The resulting solution was stirred overnight, the solvent was removed in vacuo and the residue was purified on an RP-C18 column ($H_2O$/MeOH 20:0→9:1, stepwise gradient) yielding **6** (12.0 mg, quant).

$[\alpha]_D$ −0.12 ($c$ 1.00, MeOH); $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 3.07 (t, $J$ = 11.1 Hz, 1H, H-1ax), 3.38 (m, 1H, H-5), 3.50 (dd, $J$ = 3.3, 9.9 Hz, 1H, H-3), 3.62–3.72 (m, 3H, H-2, H-6), 3.83 (d, $J$ = 3.0 Hz, 1H, H-4), 3.95 (dd, $J$ = 5.4, 11.2 Hz, 1H, H-1eq); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 60.6 (C-2), 62.9 (C-6), 69.1 (C-1), 70.6 (C-4),

75.3 (C-3), 81.0 (C-5); HR-MS Calcd for $C_6H_{11}N_3NaO_4$ $[M+Na]^+$: 212.0647; Found 212.0650.

#### 4.1.6. Ethyl 4-(2-propynyloxy)-benzoate (10e)

Ethyl 4-hydroxybenzoate (**8**, 500 mg, 3.01 mmol) was dissolved in dry acetone (5 mL), and $K_2CO_3$ (580 mg, 4.21 mmol) was added, followed by propargyl bromide (651 μL, 6.02 mmol), and the mixture was refluxed for 3 h. The mixture was then diluted with DCM (50 mL), washed with $H_2O$ (25 mL) and brine (25 mL), dried ($Na_2SO_4$), and the solvent was removed in vacuo. The residue was purified by flash chromatography on silica gel (petrol ether/EtOAc 20:1→4:1), yielding **10e** (452 mg, 73%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.36 (t, $J$ = 7.2 Hz, 3H, $OCH_2CH_3$), 2.55 (t, $J$ = 2.4 Hz, 1H, CH), 4.33 (q, $J$ = 7.2 Hz, 2H, $OCH_2CH_3$), 4.73 (d, $J$ = 2.4 Hz, 2H, $CH_2$, propynyl), 6.98, 8.00 (AA′, BB′ of AA′BB′, $J$ = 9.0 Hz, 4H, $C_6H_4$); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 14.3 ($OCH_2CH_3$), 55.7 ($OCH_2$), 60.5 ($OCH_2CH_3$), 76.0 (CH), 77.8 ($C_Q$), 114.4, 123.7, 131.4, 161.0 (6C, $C_6H_4$), 166.2 (CO); Anal. Calcd for $C_{12}H_{12}O_3$: C, 70.58; H, 5.92. Found: C, 70.58; H, 5.93.

#### 4.1.7. 1-Trifluoromethylsulfonamido-4-(2-propynyloxy)benzene (10f)

Compound **9**[37] (100 mg, 679 μmol) was dissolved in dry DCM (3 mL). Triethylamine (103 μL, 747 μmol) was added, followed by the dropwise addition of triflic anhydride (123 μL, 747 μmol) at 0 °C. The solution was allowed to reach rt and stirred under argon overnight. The solvent was removed in vacuo and the residue was purified by flash chromatography on silica gel (petrol ether/EtOAc 19:1→9:1), yielding **10f** (130 mg, 69%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 2.54 (t, $J$ = 2.4 Hz, 1H, CH); 4.69 (d, $J$ = 2.4 Hz, 2H, $OCH_2$); 6.97, 7.23 (AA′, BB′ of AA′BB′, $J$ = 8.9 Hz, 4H, $C_6H_4$); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 56.1 ($OCH_2$), 76.1 (CH), 77.9 ($C_Q$), 119.8 ($J$ = 322.8 Hz, $CF_3$), 115.8, 126.6, 126.9, 157.2 ($C_6H_4$); Anal. Calcd for $C_{10}H_8NO_3F_3S$: C, 43.01; H, 2.89; N, 5.02. Found: C, 42.95; H, 3.00; N, 4.97.

#### 4.1.8. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-phenoxymethyl-1,2,3-triazole (11a)

Compound **7** (50.0 mg, 158 μmol) was dissolved in dry DCM (3 mL). The mixture was degassed in an ultrasound bath under a flow of argon for 20 min. Copper(I) chloride (31.2 mg, 316 μmol), DIPEA (54.4 μL, 316 μmol) and **10a** (40.6 μL, 316 μmol) were added. The mixture was stirred under argon at rt for 24 h. The solvent was removed in vacuo, and the crude mixture was purified by flash chromatography (petrol ether/EtOAc 3:2) to yield compound **11a** (66.5 mg, 93%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.81, 2.08, 2.19 (3s, 9H, 3 OAc), 4.00–4.07 (m, 2H, H-1ax, H-5), 4.15 (d, $J$ = 6.4 Hz, 2H, H-6), 4.33 (dd, $J$ = 5.1, 11.6 Hz, 1H, H-1eq), 4.92 (dt, $J$ = 5.0, 11.0 Hz, 1H, H-2), 5.22 (s, 2H, $CH_2$OPh), 5.50 (dd, $J$ = 3.2, 11.0 Hz, 1H, H-3), 5.54 (d, $J$ = 2.5 Hz, 1H, H-4), 6.95–6.99, 7.26–7.30 (m, 5H, $C_6H_5$), 7.62 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.3, 20.7, 20.7 (3 $COCH_3$), 56.0 (C-2), 61.8 (2C, $CH_2$OPh, C-6), 67.0 (C-4), 68.7 (C-1), 71.5 (C-3), 75.3 (C-5), 114.7, 121.4, 122.7, 129.6 (6C, $C_6H_5$), 118.5 (C-5 triazole), 144.3 (C-4 triazole), 169.3, 170.0, 170.5 (3 CO); HR-MS Calcd for $C_{21}H_{26}N_3O_8$ $[M+H]^+$: 448.1720; Found: 448.1725.

#### 4.1.9. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-(4-cyanophenoxy)methyl-1,2,3-triazole (11b)

Prepared according to the procedure described for **11a**, using **7** (50.0 mg, 158 μmol) **10b**[38] (49.7 mg, 316 μmol). The compound was purified by flash chromatography (petrol ether/EtOAc 1:1→0:1) to give **11b** (73.0 mg, 98%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.79, 2.04, 2.16 (3s, 9H, 3 $COCH_3$), 3.99–4.04 (m, 2H, H-1ax, H-5), 4.12 (d, $J$ = 6.4 Hz, 2H, H-6), 4.30 (dd, $J$ = 5.0, 11.6 Hz, 1H, H-1eq), 4.92 (dt, $J$ = 4.9, 10.7 Hz, 1H, H-2), 5.22 (s, 2H, $CH_2$OAr), 5.48–5.52 (m, 2H, H-3, H-4), 7.01, 7.56 (m, 4H, $C_6H_4$), 7.67 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.2, 20.5, 20.6 (3 $COCH_3$), 56.1 (C-2), 61.7, 61.9 (C-6, $CH_2$OAr), 66.9 (C-4), 68.6 (C-1), 71.3 (C-3), 75.2 (C-5), 115.4 (2C, 2 C-*ortho*), 118.8 (C-*para*), 123.0 (C-5 triazole), 134.0 (2C, 2 C-*meta*), 142.9 (C-4 triazole), 161.2 (C-*ipso*), 169.2, 169.8, 170.4 (3 CO); HR-MS Calcd for $C_{22}H_{24}N_4O_8$ $[M+H]^+$: 473.1672; Found: 473.1667.

#### 4.1.10. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-(4-nitrophenyloxy) methyl-1,2,3-triazole (11c)

Prepared according to the procedure described for **11a**, using **7** (50.0 mg, 158 μmol) and **10c**[37] (49.7 mg, 316 μmol). The compound was purified by flash chromatography (petrol ether/EtOAc 1:1) to give **11c** (74.0 mg, 95%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.80, 2.03, 2.15 (3s, 9H, 3 $COCH_3$), 3.99–4.04 (m, 2H, H-1ax, H-5), 4.12 (d, $J$ = 6.4 Hz, 2H, H-6), 4.30 (dd, $J$ = 5.0, 11.6 Hz, 1H, H-1eq), 4.93 (m, 1H, H-2), 5.26 (s, 2H, $CH_2$OAr), 5.49–5.52 (m, 2H, H-3, H-4), 7.02 (AA′ of AA′BB′, $J$ = 9.3 Hz, 2H, $C_6H_4$), 7.70 (s, 1H, H-5 triazole), 8.15 (BB′ of AA′BB′, $J$ = 9.3 Hz, 2H, $C_6H_4$); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.3, 20.6, 20.7 (3 $COCH_3$), 56.2 (C-2), 61.9, 62.3 (C-6, $CH_2$OAr), 67.0 (C-4), 68.6 (C-1), 71.4 (C-3), 75.3 (C-5), 114.8 (2C, 2 C-*ortho*), 123.2 (C-5 triazole), 125.9 (2C, 2 C-*meta*), 141.9 (C-*para*), 142.8 (C-4 triazole), 163.0 (C-*ipso*), 169.3, 170.0, 170.5 (3 CO); HR-MS Calcd for $C_{21}H_{24}N_4O_{10}$ $[M+H]^+$: 493.1571; Found: 493.1579.

#### 4.1.11. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-(pentafluorophenoxy) methyl-1,2,3-triazole (11d)

Prepared according to the procedure described for **11a**, using **7** (50.0 mg, 158 μmol) and **10d**[39] (70.2 mg, 316 μmol). The compound was purified by flash chromatography (petrol ether/EtOAc 4:1→1:1) to give **11d** (62.0 mg, 73%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.85, 2.05, 2.18 (3s, 9H, 3 $COCH_3$), 3.97–4.02 (m, 2H, H-1ax, H-5), 4.13 (d, $J$ = 6.4 Hz, 2H, H-6), 4.29 (dd, $J$ = 5.0, 11.6 Hz, 1H, H-1eq), 4.95 (dt, $J$ = 5.0, 11.0 Hz, 1H, H-2), 5.27 (s, 2H, $CH_2$OAr), 5.49–5.53 (m, 2H, H-3, H-4), 7.74 (m, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.1, 20.5, 20.6 (3 $COCH_3$), 56.1 (C-2), 61.8 (C-6), 66.9 (C-4), 67.6 ($CH_2$OAr), 68.6 (C-1), 71.3 (C-3), 75.2 (C-5), 123.6 (C-5 triazole), 136.9, 139.0, 141.1, 142.9 (6C, $C_6F_5$), 142.5 (C-4 triazole), 169.3, 169.9, 170.4 (3 CO); HR-MS Calcd for $C_{21}H_{21}F_5N_3O_8$ $[M+H]^+$: 538.1249; Found: 538.1252.

#### 4.1.12. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-(4-ethoxycarbonylphenoxy)methyl-1,2,3-triazole (11e)

Prepared according to the procedure described for **11a**, using **7** (50.0 mg, 160 μmol) and **10e** (64.5 mg, 316 μmol). The compound was purified by flash chromatography (hexane/EtOAc 3:2→2:3) to give **11e** (62.0 mg, 82%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.35 (t, $J$ = 7.1 Hz, 3H, $CH_2CH_3$), 1.79, 2.05, 2.16 (3s, 9H, 3 $COCH_3$), 3.99–4.10 (m, 2H, H-1ax, H-5), 4.13 (d, $J$ = 6.4 Hz, 2H, H-6), 4.29–4.34 (m, 3H, H-1eq, $CH_2CH_3$), 4.92 (dt, $J$ = 4.9, 10.9 Hz, 1H, H-2), 5.23 (s, 2H, $CH_2$OAr), 5.49–5.53 (m, 2H, H-3, H-4), 6.95 (AA′ of AA′BB′, $J$ = 8.9 Hz, 2H, $C_6H_4$), 7.66 (s, 1H, H-5 triazole), 7.96 (BB′ of AA′BB′, $J$ = 8.9 Hz, 2H, $C_6H_4$); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 14.3 ($CH_2CH_3$), 20.2, 20.5, 20.6 (3 $COCH_3$), 56.0 (C-2), 60.6 ($CH_2CH_3$), 61.8 (2C, C-6, $CH_2$OAr), 66.9 (C-4), 68.6 (C-1), 71.4 (C-3), 75.2 (C-5), 114.2 (2C, 2 C-*ortho*), 122.9 (C-5 triazole), 131.5 (2C, 2 C-*meta*), 131.6 (C-*para*), 143.5 (C-4 triazole), 161.5 (C-*ipso*), 169.2, 169.9, 170.4 (3 CO); HR-MS Calcd for $C_{24}H_{30}N_3O_{10}$ $[M+H]^+$: 520.1931; Found: 520.1937.

#### 4.1.13. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-(trifluoromethylsulfonamidophenyloxy)methyl-1,2,3-triazole (11f)

Prepared according to the procedure described for **11a**, using **7** (50.0 mg, 158 μmol) and **10f** (88.2 mg, 316 μmol). The compound was purified by flash chromatography (petrol ether/EtOAc 3:1→1:1) to give **11f** (39.0 mg, 41%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.81, 2.06, 2.18 (3s, 9H, 3 COC$H_3$), 4.00–4.05 (m, 2H, H-1ax, H-5), 4.14 (d, $J$ = 6.4 Hz, 2H, H-6), 4.32 (dd, $J$ = 5.0, 11.6 Hz, 1H, H-1eq), 4.95 (m, 1H, H-2), 5.12 (s, 2H, C$H_2$OAr), 5.50–5.53 (m, 2H, H-3, H-4), 6.88, 7.22 (AA′, BB′ of AA′BB′, $J$ = 8.8 Hz, 4H, $C_6H_4$), 7.68 (s, 1H, H-5 triazole), 8.56 (s, 1H, NH); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.2, 20.6, 20.7 (3 COCH$_3$), 56.3 (C-2), 61.7, 61.9 (C-6, C$H_2$OAr), 67.0 (C-4), 68.6 (C-1), 71.4 (C-3), 75.2 (C-5), 115.4 (2C, 2 C-*ortho*), 119.84 (q, $J$ = 323.1 Hz, $CF_3$), 123.2 (C-5 triazole), 126.6 (2C, 2 C-*meta*), 127.1 (C-*para*), 143.5 (C-4 triazole), 157.3 (C-*ipso*), 169.5, 170.1, 170.6 (3 CO); HR-MS Calcd for $C_{22}H_{25}F_3N_4NaO_{10}$ $[M+Na]^+$: 617.1141; Found: 617.1150.

#### 4.1.14. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-(4-bromophenoxy) methyl-1,2,3-triazole (11g)

Prepared according to the procedure described for **11a**, using **7** (200 mg, 634 μmol) and **10g**[40] (267 mg, 1.07 mmol). The compound was purified by flash chromatography (hexane/EtOAc 1:1) to give **11g** (279 mg, 84%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.74, 2.00, 2.11 (3s, 9H, 3 COCH$_3$), 3.93–4.00 (m, 2H, H-1ax, H-5), 4.07 (d, $J$ = 6.4 Hz, 2H, H-6), 4.24 (dd, $J$ = 5.0, 11.6 Hz, 1H, H-1eq), 4.86 (dt, $J$ = 5.0, 11.0 Hz, 1H, H-2), 5.10 (s, 2H, C$H_2$OAr), 5.43–5.47 (m, 2H, H-3, H-4), 6.77, 7.30 (AA′, BB′ of AA′BB′, $J$ = 8.9 Hz, 4H, $C_6H_4$), 7.57 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.2, 20.5, 20.6 (3 COCH$_3$), 56.0 (C-2), 61.8, 61.9 (C-6, C$H_2$OAr), 66.9 (C-4), 68.6 (C-1), 71.4 (C-3), 75.2 (C-5), 113.5 (C-Br), 116.5 (2C, 2 C-*ortho*), 122.8 (C5 triazole), 132.3 (2C, 2 C-*meta*), 143.7 (C-4 triazole), 157.0 (C-*ipso*), 169.2, 169.9, 170.4 (3 CO); HR-MS Calcd for $C_{21}H_{25}BrN_3O_8$ $[M+H]^+$: 526.0825; Found: 526.0818.

#### 4.1.15. 1-(1,5-Anhydro-2-deoxy-D-galactitol-2-yl)-phenoxymethyl-1,2,3-triazole (1a)

Compound **11a** (65.0 mg, 145 μmol) was dissolved in dry MeOH (5 mL) and sodium metal (20.0 mg, 869 μmol) was added. The solution was stirred at rt under argon for 3 h, after which the solvent was removed in vacuo and the residue was purified by preparative LC-MS to give **1a** (21.0 mg, 45%).

$[\alpha]_D$ +36.4 ($c$ 0.17, MeOH); $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 3.31 (t, $J$ = 1.6 Hz, 1H, H-5), 3.61–3.81 (m, 2H, H-6), 3.84 (t, $J$ = 11.1 Hz, 1H, H-1ax), 3.99 (d, $J$ = 3.0 Hz, 1H, H-4), 4.14–4.18 (m, 2H, H-1eq, H-3), 4.81 (dt, $J$ = 5.0, 10.8 Hz, 1H, H-2), 5.16 (s, 2H, C$H_2$OPh), 6.93–7.01, 7.26–7.30 (m, 5H, $C_6H_5$), 8.14 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 60.7 (C-2), 62.3 (C$H_2$OPh), 62.9 (C-6), 69.8 (C-1), 70.4 (C-4), 73.5 (C-3), 81.5 (C-5), 122.2, 125.8, 130.6, 159.8 (6C, $C_6H_5$), 115.9 (C-5 triazole), 144.53 (C-4 triazole); HR-MS Calcd for $C_{15}H_{20}N_3O_5$ $[M+H]^+$: 322.1403; Found: 322.1401.

#### 4.1.16. 1-(1,5-Anhydro-2-deoxy-D-galactitol-2-yl)-4-(4-cyanophenoxy)methyl-1,2,3-triazole (1b)

Compound **11b** (73.0 mg, 155 μmol) was dissolved in $H_2O$/MeOH/$Et_3N$ (5:5:1, 5.5 mL) and stirred at rt overnight. The solvent was removed in vacuo and the residue was purified by flash chromatography (DCM/MeOH 9:1) to give **1b** (44.0 mg, 81%).

$^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 3.62 (m, 1H, H-5), 3.73 (dd, $J$ = 4.9, 11.4 Hz, 1H, H-6a), 3.80 (dd, $J$ = 7.1, 11.4 Hz, 1H, H-6b), 3.84 (t, $J$ = 11.1 Hz, 1H, H-1ax), 3.99 (d, $J$ = 3.0 Hz, 1H, H-4), 4.14–4.18 (m, 2H, H-1eq, H-3), 4.82 (dt, $J$ = 5.0, 10.9 Hz, 1H, H-2), 5.36 (s, 2H, C$H_2$OAr), 7.17, 7.67 (AA′, BB′ of AA′BB′, $J$ = 9.0 Hz, 4H, $C_6H_4$), 8.19 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 60.7 (C-2), 62.6 (C$H_2$OAr), 62.9 (C-6), 69.7 (C-1), 70.3 (C-4), 73.5 (C-3), 80.5 (C-5), 105.3 (CN), 116.8 (2C, 2 C-*ortho*), 120.0 (C-*para*), 126.2 (C-5 triazole), 135.2 (2C, 2 C-*meta*), 143.5 (C-4 triazole), 163.2 (C-*ipso*); HR-MS Calcd for $C_{16}H_{19}N_4O_5$ $[M+H]^+$: 347.1355; Found: 347.1358.

#### 4.1.17. 1-(1,5-Anhydro-2-deoxy-D-galactitol-2-yl)-4-(4-nitrophenoxy)methyl-1,2,3-triazole (1c)

Compound **11c** (74.0 mg, 150 μmol) was deacetylated according to the procedure described for **1a**. The final product was purified by LC-MS to give **1c** (18.0 mg, 33%).

$^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 3.76–3.87 (m, 2H, H-5, H-6a), 3.90 (t, $J$ = 11.2 Hz, 1H, H-1ax), 4.10 (m, 2H, H-6b, H-4), 4.24 (dd, $J$ = 5.1, 11.2 Hz, 1H, H-1eq), 4.28 (dd, $J$ = 3.2, 10.6 Hz, 1H, H-3), 4.87 (m, 1H, H-2), 5.38 (s, 2H, C$H_2$OAr), 7.23 (AA′ of AA′BB′, $J$ = 9.3 Hz, 2H, $C_6H_4$), 8.29 (m, 3H, $C_6H_4$, H-5 triazole); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 60.3 (C-2), 62.5, 62.6 (C-6, C$H_2$OAr), 69.3 (C-1), 69.8 (C-4), 72.9 (C-3), 81.0 (C-5), 116.1 (2C, 2 C-*ortho*), 126.5 (C-5 triazole), 127.0 (2C, 2 C-*meta*), 142.6 (C-*para*), 143.3 (C-4 triazole), 164.5 (C-*ipso*); HR-MS Calcd for $C_{15}H_{19}N_4O_7$ $[M+H]^+$: 367.1254; Found: 367.1255.

#### 4.1.18. 1-(1,5-Anhydro-2-deoxy-D-galactitol-2-yl)-4-(pentafluorophenoxy)methyl-1,2,3-triazole (1d)

Compound **11d** (60.0 mg, 111 μmol) was deacetylated according to the procedure described for **1a**. The crude product was purified by LC-MS to give **1d** (31.0 mg, 68%).

$^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 3.59 (m, 1H, H-5), 3.69 (dd, $J$ = 4.9, 11.4 Hz, 1H, H-6a), 3.75-3.90 (m, 2H, H-1ax, H-6b), 3.97 (m, 1H, H-4), 4.10-4.14 (m, 2H, H-1eq, H-3), 4.78 (dt, $J$ = 5.0, 10.8 Hz, 1H, H-2), 5.20 (m, 2H, C$H_2$OAr), 8.10 (m, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 60.7 (C-2), 62.9 (C-6), 68.4 (C$H_2$OAr), 66.5 (C-1), 70.4 (C-4), 73.5 (C-3), 81.5 (C-5), 126.9 (C-5 triazole), 142.9 (C-4 triazole); HR-MS Calcd for $C_{15}H_{15}F_5N_3O_5$ $[M+H]^+$: 412.0932; Found: 412.0934.

#### 4.1.19. 1-(1,5-Anhydro-2-deoxy-D-galactitol-2-yl)-4-(sodium 4-carboxyphenoxy)methyl-1,2,3-triazole (1e)

Compound **11e** (62.0 mg, 119 μmol) was dissolved in MeOH/$H_2O$ (4 mL, 1:1), and NaOH (170 mg, 4.72 mmol) was added. The solution was stirred at rt for 24 h, after which the solvent was removed in vacuo, and the residue was purified by flash chromatography (DCM/MeOH 5:1→2:1) to give **1e** (32.0 mg, 74%).

$^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 3.62 (m, 1H, H-5), 3.73 (m, 2H, H-6), 3.81 (t, $J$ = 11.3 Hz, 1H, H-1ax), 3.99 (d, $J$ = 3.0 Hz, 1H, H-4), 4.11-4.17 (m, 2H, H-3, H-1eq), 4.80 (m, 1H, H-2), 5.20 (s, 2H, C$H_2$OAr), 7.02, 7.93 (AA′, BB′ of AA′BB′, $J$ = 8.8 Hz, 4H, $C_6H_4$), 8.19 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 60.6 (C-2), 62.4 (C$H_2$OAr), 62.9 (C-6), 69.7 (C-4), 70.5 (C-1), 73.3 (C-3), 81.2 (C-5), 115.1 (2C, 2 C-*ortho*), 126.0 (C-5 triazole), 127.5 (C-*para*), 132.5 (2C, 2 C-*meta*), 144.1 (C-4 triazole), 162.8 (C-*ipso*), 173.7 (CO); HR-MS Calcd for $C_{16}H_{19}N_3NaO_7$ $[M+H]^+$: 388.1121; Found: 388.1128.

#### 4.1.20. 1-(1,5-Anhydro-2-deoxy-D-galactitol-2-yl)-4-(4-trifluoromethylsulfonamidophenoxy) methyl-1,2,3-triazole (1f)

Compound **11f** (39.0 mg, 61.6 μmol) was deacetylated according to the procedure described for **1a**. The final product was purified by flash chromatography on silica gel (DCM/MeOH 10:1) to give **1f** (21.2 mg, 73%).

$^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 3.58 (m, 1H, H-5), 3.68 (dd, $J$ = 4.8, 11.4 Hz, 1H, H-6a), 3.73–3.81 (m, 2H, H-1ax, H-6b), 3.95 (d, $J$ = 3.0 Hz, 1H, H4), 4.10–4.13 (m, 2H, H-1eq, H-3), 4.77 (dt, $J$ = 5.1, 10.8 Hz, 1H, H-2), 5.11 (s, 2H, C$H_2$OAr), 6.94, 7.13 (AA′ of AA′BB′, $J$ = 9.0 Hz, 2H, $C_6H_4$), 8.11 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 60.7 (C-2), 62.6 (C$H_2$OAr), 62.9 (C-6), 69.7

(C-1), 70.4 (C-4), 73.4 (C-3), 81.4 (C-5), 116.3 (2C, 2 C-*ortho*), 121.9 (q, $J$ = 323.9 Hz, $CF_3$), 125.9 (C-5 triazole), 126.8 (2C, 2 C-*meta*), 131.4 (C-*para*), 144.3 (C-4 triazole), 158.0 (C-*ipso*); HR-MS Calcd for $C_{16}H_{19}F_3N_4O_7SNa$ $[M+Na]^+$: 491.0824; Found: 491.0828.

#### 4.1.21. 1-(3,4,6-Tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-galactitol-2-yl)-4-[(4-methyl-piperazin-1-yl)-phenoxymethyl]-1,2,3-triazole (11h)

Compound **11g** (75.0 mg, 142 μmol) and $Cs_2CO_3$ (64.8 mg, 199 μmol) were azeotropically dried with toluene, and $Pd_2dba_3$ (3.00 mg, 2.89 μmol), X-phos[42] (**12**, 1.35 mg, 2.84 μmol), *N*-methylpiperazine (17.0 μL, 157 μmol) and dry toluene (3 mL) were added. The resultant mixture was stirred at 80 °C under argon for 24 h. The solvent was removed in vacuo, and the residue purified by flash chromatography (EtOAc/MeOH 9:1→4:1) to give **11h**, which was still slightly impure according to NMR. The compound was used without further purification in the next step.

#### 4.1.22. 1-(1,5-Anhydro-2-deoxy-D-galactitol-2-yl)-4-[(4-methyl-piperazin-1-yl)-phenoxymethyl]-1,2,3-triazole (1h)

Compound **11h** (35.1 mg, 64.3 μmol) was deacetylated according to the procedure described for **1a**. The final product was purified by flash chromatography (DCM/MeOH 9:1→7:3) to give **1h** (12.0 mg, 44%).

$^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 2.63 (s, 3H, $NCH_3$), 3.02 (m, 4H, ($CH_2$)$_2$NCH$_3$), 3.18 (m, 4H, ($CH_2$)$_2$NPh), 3.59 (m, 1H, H-5), 3.69 (dd, $J$ = 4.9, 11.4 Hz, 1H, H-6a), 3.74–3.82 (m, 2H, H-1ax, H-6b), 3.95 (d, $J$ = 3.0 Hz, 1H, H-4), 4.10-4.13 (m, 2H, H-1eq, H-3), 4.02 (dd, $J$ = 3.0, 10.5 Hz, 1H, H-3), 4.76 (m, 1H, H-2), 5.07 (s, 2H, $CH_2$OAr), 6.92 (m, 4H, $C_6H_4$), 8.09 (s, 1H, H-5 triazole); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 44.7 ($NCH_3$), 49.5 (2C, ($CH_2$)$_2$NCH$_3$), 55.4 (2C, ($CH_2$)$_2$NPh), 60.7 (C-2), 62.8 (2C, C-6, $CH_2$OAr), 69.7 (C-1), 70.4 (C-4), 73.4 (C-3), 81.4 (C-5), 116.7 (2C, 2 C-*ortho*), 119.9 (2C, 2 C-*meta*), 125.8 (C-5 triazole), 144.6 (C-*para*), 146.4 (C-4 triazole), 154.7 (C-*ipso*); HR-MS Calcd for $C_{20}H_{30}N_5O_5$ $[M+H]^+$: 420.2247; Found: 420.2252.

### 4.2. Expression and purification of recombinant H1-CRD

The vector pET3b encoding the amino acid residues 147–291 of the H1-subunit was transformed into *E. coli* strain AD494 (DE3). Following IPTG (0.4 mM) induction, the protein was accumulated as inclusion bodies. After collection and lysis of the cells the protein extract was denatured under reducing conditions and dialyzed against Tris/HCl buffer (20 mM TrisCl, 120 mM NaCl, 20 mM $CaCl_2$, pH 7.8). The correctly folded H1-CRD was purified by affinity chromatography on a D-Gal-Sepharose column attached to an FPLC-system (Bio-Rad). In a second step the monomer and dimer fractions were separated by ion exchange HPLC (Agilent 1100) on a DEAE column. The monomeric fraction was quantified by a Bradford assay[55] and immediately used for the binding assay or stored at −20 °C. The purity and identity of the purified protein were verified by SDS–PAGE and immuno-blot analysis, respectively. For the immuno-blot analysis, polyclonal (IgY) anti-H1-CRD antibodies were used. The antibodies were produced in chicken and purified from their eggs after PEG-precipitation[56] by affinity chromatography on a agarose-immobilized H1-CRD column.

### 4.3. Competitive solid-phase binding assay

Flat-bottom 96-well microtiter plates were coated with 100 μL/well of a 3 μg/mL solution of monomeric H1-CRD in 20 mM HEPES, 150 mM NaCl and 1 mM $CaCl_2$, pH 7.4 (HBS-buffer) overnight at 4 °C. The coating solution was discarded and the wells were blocked with 150 μL/well of 1% BSA in HBS-buffer for 2 h at 4 °C. After three washing steps with 150 μL/well of HBS-buffer, 50 μL/well of the test compound solution (6 μM to 2 mM) and 50 μL of a 0.5 μg/mL of streptavidin-peroxidase coupled PAA-glycopolymers (D-Gal*N*Ac-, D-Gal-PAA) were added. The plates were incubated for 2 h at rt and 250 rpm. The plates were then carefully washed twice with 150 μL/well HBS-buffer. After the addition of 100 μL/well of ABTS-substrate, the colorimetric reaction was allowed to develop for 2 min. The reaction was stopped by the addition of 2% aqueous oxalic acid and the optical density (OD) was measured at 415 nm on a microplate-reader (Spectramax 190, Molecular Devices, California, USA). The $IC_{50}$ values of the compounds tested in triplicates in three independent experiments were calculated with PRISM software (GraphPad Software, Inc, La Jolla, USA). The data set was normalized to percentage of binding by setting background binding (D-Gal*N*Ac-PAA binding to wells without H1-CRD) as 0% and control binding (maximal D-Gal*N*Ac-PAA binding to H1-CRD) as 100%. The nonlinear fit of the normalized data was calculated with a four-parameter logistic equation with the bottom of the curve constraint to zero and the top to 100. The $IC_{50}$ defines the molar concentration of the test compound that reduces the maximal specific binding of D-Gal*N*Ac-PAA to H1-CRD by 50%. The relative $IC_{50}$ ($rIC_{50}$) is the ratio of the $IC_{50}$ of the test compound to the $IC_{50}$ of D-Gal*N*Ac.

### 4.4. Surface Plasmon Resonance Experiments (Biacore)

The analyses were performed on a Biacore 3000 machine (Biacore AB, Uppsala, Sweden) using CM5 chips. After activating the surface of the chip for 5–10 min at a flow rate of 5 μL/min using a standard amine coupling procedure, H1-CRD, diluted in 10 mM acetate buffer pH 4.5 to a final concentration of 20 μg/mL, was injected for 5–15 min and the surface was deactivated for a time corresponding to the activation phase. The obtained surface densities ranged from 1800 to 2500 RUs. HBS-N buffer (10 mM HEPES pH 7.4, 50 mM $CaCl_2$) was used as running buffer. The screening of the compounds was performed by the injection of twofold serial dilutions between 5 mM and 5 μM as randomized triplicates. Each sample was injected for 30 s with an undisturbed dissociation phase of 20 s and 50 μL/min. No regeneration or washing steps were applied. Five buffer blanks were injected at the beginning and one between the triplicate series. Signals of an untreated flow cell and averaged blank injections were subtracted from the sample sensorgrams. Since referenced sensorgrams showed negative SPR signals, the whole data set was mirrored by multiplication of each data point with −1. Mirrored steady state data were evaluated between 10 and 20 s of the injection period and were fitted to a single site-binding model. Data processing and equilibrium binding constant determinations were accomplished with SCRUBBER software Version 2.

### 4.5. NMR experiments

Shigemi NMR tubes were used to reduce the sample volume, and therefore protein consumption, needed for measurement to 250 μL. The ASGP-R protein was present at 17 μM, as determined by a Bradford assay. The buffer consisted of 1 mM $CaCl_2$ in $D_2O$ (Armar Chemicals), maintained at pH 7.5 with 25 mM Tris-$d_{11}$. A stock solution of D-Gal*N*Ac was prepared in $D_2O$ at 50 mM. A stock solution of **1b** in DMSO-$d_6$ was prepared at 50 mM.

All NMR experiments were carried out at 300 K on a Bruker DRX500 spectrometer, equipped with *Z*-gradient SEI probe. The pulse sequence used for the T1rho relaxation was slightly modified from that described by Hajduk.[57] Following the 90° non-selective pulse, a continuous-wave spin-lock of 2 kHz was applied for a various duration to monitor the T1rho relaxation. The pulse sequence terminated with the DPFGSE water suppression sequence to suppress the magnetization from the residual protons in the $D_2O$

solvent.[58] For each T1rho time constant measurement, 15 experiments were performed with different durations of the spin-lock which started at 20 ms to a final value of 300 ms, in intervals of 20 ms. For concentrations of D-Gal*N*Ac that were less than 600 μM, 16 scans were measured, preceded by eight dummy scans, and a recovery delay of 10 s between successive scans. For concentrations of D-Gal*N*Ac that were 600 μM or greater, the identical procedure was followed, except that only eight scans were measured. Prior to the measurement upon addition of a competing compound, a 1 h incubation time for equilibration was allowed.

The NMR data were analyzed using XWINNMR version 3.5 operating on a PC running under Linux OS. The spectra were apodized with an exponential decay function with 2 Hz line broadening. The inversion recovery data, as well as the one-site binding model, were fit using PRISM 4 (GraphPad Software Inc., San Diego, USA).

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## Supplementary data

Supplementary data ($^1$H NMR spectra of target compounds **1a**–**f**, **1h** and **6**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.049.

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