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Synthesis of a 3′-naphthamido-LacNAc fluorescein conjugate with high selectivity and affinity for galectin-3

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Abstract—Described is the synthesis of a fluorescent LacNAc derivative appended with a 3′-deoxy-3′-naphthamido functionality, 2-(fluorescein-5/6-amido)ethyl 3-deoxy-3-(2-naphthamido)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside, which confers high affinity (K_d 170 nM) and selectivity for galectin-3 via a stacking interaction with Arg144. Its use as a selective and sensitive galectin-3 probe is demonstrated with fluorescence polarization measurements.

Keywords: Galectin; Lectin; Fluorescence polarization; Cation–π

1. Introduction

The galectins are a family of 14 soluble β-D-galactose-binding[1,2] cytosolic proteins that have been implicated to play important roles in a range of biological events[3–11] of which cancer,[6,7,10,12] inflammation and immune regulation[5,13–15] are the most apparent. However, many questions concerning galectin function remain elusive and further research is motivated by their importance in disease-related conditions. Sensitive research tools for specific monitoring of galectin expression and activity are thus desirable. Within this context, fluorescence-based research tools and probes can play a central role. Various fluorescence-based molecular biology techniques have over the recent years evolved into powerful and sensitive means of studying protein expression and activity. Fluorescence polarization is related to molecular size and can be used to detect the binding of a small fluorescent ligand to a protein. We have recently reported the development of a highly sensitive and reliable method for studying galectin–ligand interactions based on binding and inhibition of fluorescent glycoconjugates to galectins.[16–18] Unfortunately, glycoconjugate–protein interactions are typically weak and so are galectin–ligand interactions with, at the best, low micromolar affinities for natural saccharide ligands. Thus, fluorescent glycoconjugate probes based on natural saccharide structures[16–18] with relatively low affinity for galectins have somewhat limited sensitivity in fluorescence polarization assays, as well as limited use in other galectin-detecting assays. The synthesis of fluorescent probes based on chemically modified structures with improved affinities for galectins has consequently emerged as an important task. In addition, a high-affinity fluorescent probe specific for a galectin can complement the use of fluorescent antibodies in visualizing galectin activities in vivo, for example, to detect growing tumors. A selective ligand-based fluorescent probe for detecting galectins in vivo has the advantage over galectin-antibodies in that it distinguishes ligand-binding galectins from non-binding, presumably unfolded or defective, galectins.

We have recently reported high-affinity inhibitors of galectin-3 based on LacNAc derivatives, which had aromatic amido groups at C3′, which forms a strong

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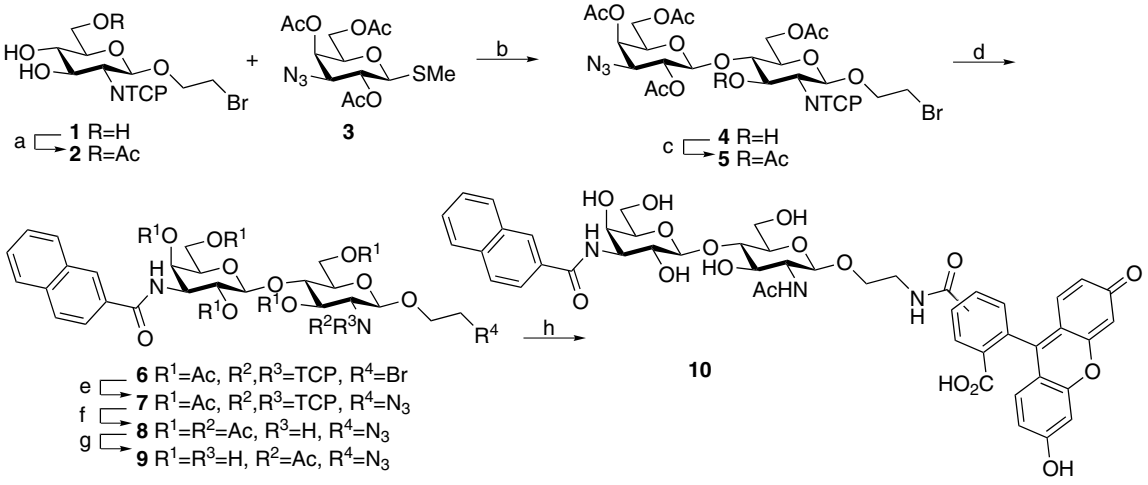
interaction with the guanidinium ion of galectin-3 Arg-144.[19,20] The most powerful affinity-enhancing aromatic amido groups at LacNAc C3′ were found to be 2-naphthamides and fluorescence-tagging of such a 3′-naphthamido LacNAc derivative was hypothesized to provide a high-affinity fluorescent probe for galectin-3. Herein, we report the synthesis of a 3′-naphthamido LacNAc derivative equipped with a fluorescein-carrying aglycon and the demonstration of its nanomolar affinity for galectin-3.

2. Results and discussion

The synthetic strategy involved the regioselective glycosylation at the C4 hydroxyl group of an N-protected glucosamine 2-bromoethyl glycoside with a 3-azido galactopyranosyl donor. The 2-bromoethyl aglycon was chosen because it can be readily transformed into a 2-aminoethylglycoside, which in turn can be conjugated with carboxy-functionalized fluorescent molecules (e.g., carboxyfluorescein).[18] The 3-azido-galactosyl donor was chosen due to its straightforward reduction–acylation to give naphthamides.[20] Thus, regioselective 6-O-acetylation[19] of 2-bromoethyl 2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside **1** with acetyl chloride and *sym*-collidine at −47 °C gave 87% of a glycosyl acceptor **2** having the 3-OH and 4-OH groups unprotected (Scheme 1). Glycosylation of **2** with the 3-azido-1-thio-galactopyranoside **3**[19] under *N*-iodosuccinimide–triflic acid promotion[21,22] gave 3′-azido LacNAc 2-bromoethyl glycoside **4** in 52%. Acetylation of the unprotected 3-OH group of **4** to give **5**, followed by catalytic hydrogenation of the azido group and naphthoylation yielded **6** in 56%. Treatment of **6** with sodium azide resulted in substitution of the primary bromide to furnish **7**. Removal of the TCP N-protecting group with 1,2-diaminoethane, N-acetylation, and de-O-acetylation with methanolic sodium methoxide afforded 2-azidoethyl glycoside of 3′-naphthamido-lacNAc **9**. Catalytic hydrogenation of **9** liberated a free amino group, which was acylated with NHS-5/6-carboxyfluorescein to give the water-soluble target fluorescein-tagged **10**.

Evaluation of **10** and the parent unsubstituted LacNAc probe **11**[18] against galectin-1, 2, 3, 4N, 4C, 7, 8C, 8N, 9C and 9N in fluorescence polarization experiments revealed a picture where the 3′-naphthamido moiety of **10** is affinity-enhancing for all galectins except galectin-8N (Fig. 1). This suggests that the 3′-naphthamido substituent of **10** provides favorable interactions and good surface complementarity to all galectins except galectin-8N. In particular, galectin-3, for which **10** was originally designed, bound **10** with an unusually high affinity (K_d 170 nM at 20 °C) as compared to the parent **11** (K_d 4.6 μM, Table 1). This observation confirms the affinity-enhancing interaction of the 3′-naphthamido substituent with galectin-3 Arg144.[20] However, the affinity of **10** for galectin-3 is significantly higher than that of the corresponding unlabeled methyl glycoside[20] (K_d 480 nM). This most likely is the result of beneficial interactions between the protein and the spacer and/or probe as earlier observed for fluorescein-tagged natural saccharides.[18]

Compound **10** also displayed strongly enhanced affinity for galectin-4C and 9C (K_d 2.9 and 3.1 μM, respectively), as compared to **11**. The effect was however less than for galectin-3, which suggests that 3′-naphthamido group of **10** is somewhat less well suited for interaction with these galectins, presumably due to the absence of an arginine, corresponding to Arg144 in galectin-3 that specifically interacts with the 3′-naphthamido group.



Scheme 1. (a) AcCl, *s*-collidine, CH_2Cl_2, 87%; (b) NIS, TfOH, CH_2Cl_2, 52%; (c) Ac_2O, pyridine, 70%; (d) (i) H_2, Pd/C, EtOH, HCl, (ii) Naphthoyl chloride, pyridine, 56%; (e) NaN_3, 15-crown-5, DMF, 98%; (f) (i) 1,2-Diaminoethane, EtOH, (ii) Ac_2O, pyridine, 90%; (g) $NaOCH_3$, CH_3OH, 70%; (h) (i) H_2, Pd/C, EtOH, HCl, (ii) NHS-5/6-carboxyfluorescein, $NaHCO_3$, DMSO, 21%.

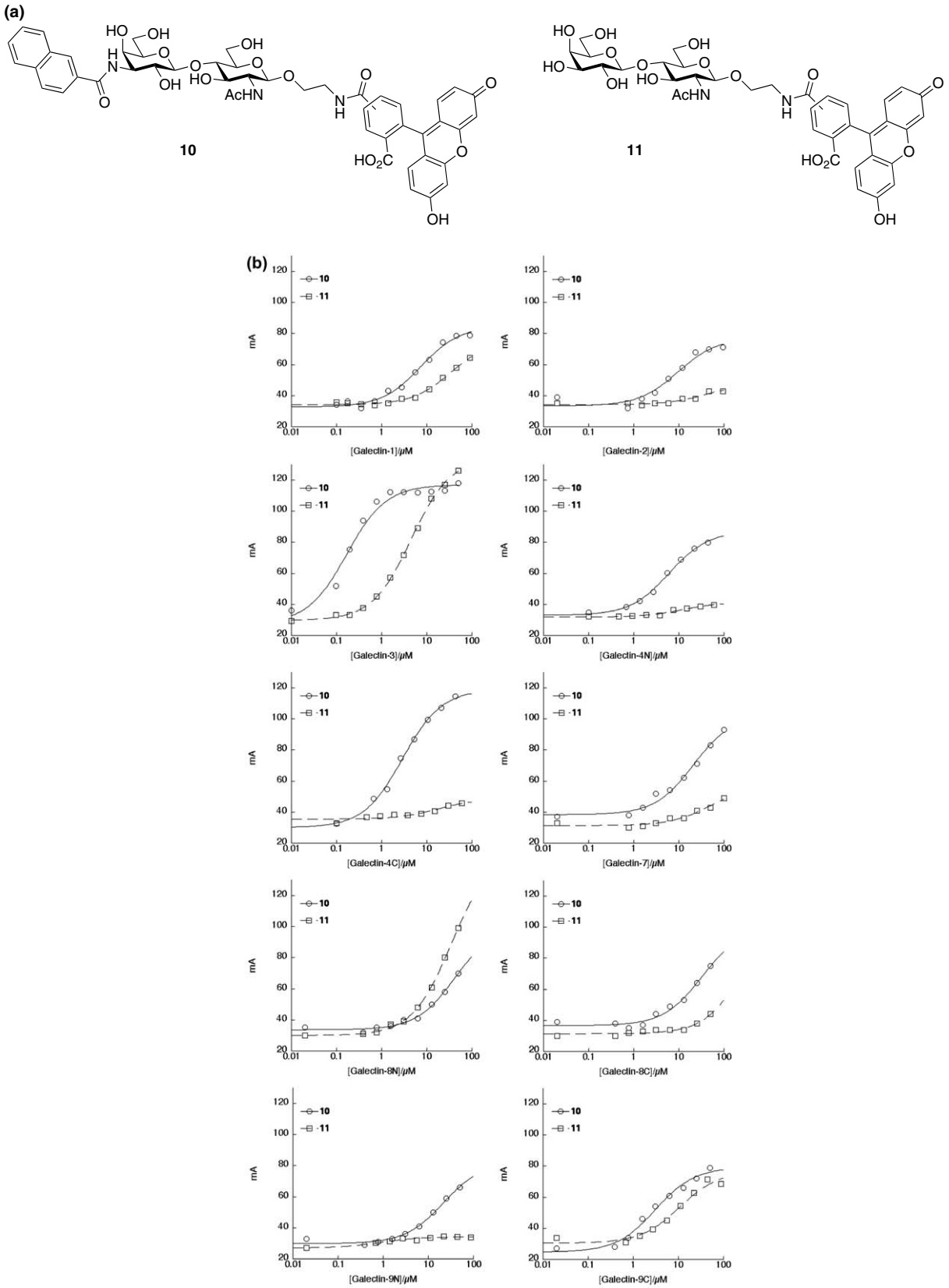


Figure 1. (a) Structure of **10** and the corresponding parent fluorescein-tagged LacNAc probe **11**. (b) The polarization values of the fluorescence-labeled probes **10** and **11** in the presence of increasing concentrations of galectins.

Table 1. Dissociation constants (μM) and maximum anisotropy values A_{max} (mA) in fluorescence polarization experiments with **10** and **11**

Galectin	T (°C)	**10** K_d[a]	**10** A_{max}	**11** K_d	**11** A_{max}
1	4	7.4 ± 1.3	85 ± 2	36 ± 6	76 ± 3
2	4	9.4 ± 3.5	77 ± 4	High	n.d.[b]
3	4	0.14 ± 0.03	99 ± 2	1.9 ± 0.3	127 ± 3
3	20	0.17 ± 0.03	117 ± 2	4.6 ± 0.2	135 ± 1
4N	4	6.1 ± 0.8	87 ± 2	High	n.d.
4C	4	2.9 ± 0.3	119 ± 2	High	n.d.
7	4	21 ± 5	103 ± 5	High	n.d.
8N	4	43 ± 12	≈100	35 ± 3	≈150
8C	4	33 ± 13	100 ± 12	High	n.d.
9N	4	22 ± 5	83 ± 5	High	n.d.
9C	4	3.1 ± 0.7	79 ± 2	10 ± 3	77 ± 3

[a] Values and standard errors were obtained by plotting anisotropy values against the galectin concentrations and fitted to the binding isotherm $A = A_0 + (A_{max} - A_0) * ([G]/(K_d + [G]))$[18] using a non-linear Levenberg–Marquart algorithm.
[b] Not determined.

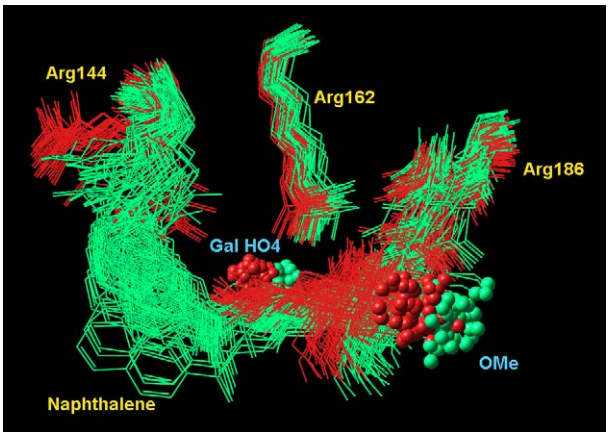


Figure 2. Overlay of conformers sampled by 100 ns molecular dynamic simulations of the methyl glycoside analogs of **10** (green) and **11** (red) in complex with galectin-3. The galactose 4-OH group and the methyl aglycon are indicated as spheres.

Interestingly, the maximum anisotropy for **10** in complex with galectin-3 is lower than for **11** (Fig. 1b), which reflects an increased movement of **10**, as compared to **11**, when bound by galectin-3. Molecular dynamic simulations of the complexes between galectin-3 and the methyl glycoside analogs of **10** and **11** suggest that the 3′-naphthamido group of **10** imposes a different clustering of conformers of the LacNAc disaccharide moiety of **10** as compared to **11** (Fig. 2). The position of the aglycon of the 3′-naphthamido-derivative **10** is, according to the simulations, clearly different than that of **11**, which could explain a difference in mobility of the fluorescein parts of **10** and **11** when bound by galectin-3.

In conclusion, attaching an unnatural 3′-naphthamido group to a fluorescein-tagged LacNAc glycoside confers improved affinity for many galectins. The 3′-naphthamido group can form an especially favorable interaction with Arg144 of galectin-3. The 3′-naphthamido LacNAc probe **10** proved to have remarkably high affinity (K_d 170 nM) for this galectin, 20-fold higher than for the two galectins (4C and 9C) with second and third highest affinity. Hence, **10** shows promising potential as a tool, not only for sensitive fluorescence polarization screening of putative inhibitors, but possibly also for selective detection and localization of galectin-3 in cells, tissue, and in vivo. The latter applications are particularly attractive in diagnosis of inflammatory conditions and cancers.

3. Experimental

3.1. General

All commercial chemicals were used without further purification, except for the following: CH_2Cl_2 for the glycoside synthesis was distilled over CaH_2 under a nitrogen atmosphere, DMF and 1,2-diaminoethane were distilled under a nitrogen atmosphere. EtOH was dried over 3 Å molecular sieves and pyridine over 4 Å molecular sieves. All reactions, except the deacetylation and the conjugation reaction, were performed under nitrogen atmosphere using syringe-septum cap techniques in oven-dried flasks flushed with nitrogen. Thin layer chromatography (TLC) was carried out on 60 F_{254} silica (Merck), detected under UV light and developed with aqueous sulfuric acid. Column chromatography (CC) was performed on silica gel (Amicon 35–70 μm, 60 Å). NMR experiments were recorded on Bruker ARX 300 MHz or Bruker DRX 400 MHz spectrometers at ambient temperature. ^{1}H NMR assignments were derived from COSY experiments. Only signals that could be unambiguously assigned are given. The optical rotations were measured with a Perkin–Elmer 341 polarimeter. MALDI-TOF MS experiments were recorded with a Bruker Biflex III instrument (run in positive mode) using gentisic acid (2,5-dihydroxy benzoic acid) as matrix. High-resolution fast atom bombardment mass spectra HRMS (FAB) were recorded with a JEOL SX-120 instrument. HPLC was performed on Beckman System Gold with solvent Module 126 (H_2O/CH_3CN), detector module 166 and column C_{18}.

3.2. 2-Bromoethyl 6-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside (1)

2-Bromoethyl 2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside[23] (0.66 g, 1.21 mmol) was dissolved in CH_2Cl_2 (20 mL) followed by the addition of *sym*-collidine (0.80 mL, 6.0 mmol). The suspension was cooled to −47 °C under a nitrogen atmosphere. Acetyl chloride (0.15 mL, 1.38 mmol) was added, the reaction mixture was stirred for 6 h and then quenched by the addition of CH_3OH (6 mL) and CH_2Cl_2 (15 mL). The organic phase was washed with aqueous HCl (20 mL, 0.5 M). The

organic layer was neutralized with satd aqueous $NaHCO_3$, dried (Na_2SO_4), filtered, and concentrated. Column chromatography (1:1, heptane/EtOAc) gave **1** (0.63 g, 87%): $[\alpha]_D^{22}$ −28 (*c* 0.9, CH_3OH); ^1H NMR (300 MHz, $CDCl_3$): δ 5.17 (d, 1H, $J = 8.4$ Hz, H-1), 4.46 (dd, 1H, $J = 4.6$, 12.2 Hz, H-6), 4.23–4.33 (m, 2H, H-3, H-6), 4.09 (dt, 1H, $J = 5.3$, 11.0 Hz, OCH_2CH_2), 4.05 (dd, 2H, $J = 8.4$, 10.9 Hz, H-2), 3.95 (d, 1H, $J = 4.8$ Hz, HO-4), 3.69–3.73 (m, 2H, HO-3, OCH_2CH_2), 3.61 (ddd, 1H, $J = 2.0$, 4.5, 9.8 Hz, H-5), 3.28–3.45 (m, 3H, H-4, OCH_2CH_2), 2.12 (s, 3H, Ac); FABMS *m*/*z* calcd for $[C_{18}H_{16}BrCl_4NNaO_8+Na]^+$: 615.8711. Found: 615.8713.

3.3. 2-Bromoethyl 2,4,6-tri-*O*-acetyl-3-azido-3-deoxy-β-D-galactopyranosyl-(1→4)-6-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside (4)

Compounds **3** (91 mg, 0.25 mmol), **1** (125 mg, 0.21 mmol) and dry CH_2Cl_2 (7 mL) were stirred over activated AW-300 molecular sieves (0.50 g) for 30 min at rt under a nitrogen atmosphere. The mixture was cooled to −42 °C and *N*-iodosuccinimide (77 mg, 0.34 mmol) was added followed by trifluoromethanesulfonic acid (3.0 μL, 34 μmol). The reaction mixture was allowed to reach rt after 80 min, and was then filtered and diluted with CH_2Cl_2. The organic layer was washed with 10% aqueous $Na_2S_2O_3$, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by chromatography (5:4, heptane/EtOAc) to give **4** (98 mg, 52%): $[\alpha]_D^{21}$ −3.4 (*c* 0.6, CH_3OH); ^1H NMR (300 MHz, $CDCl_3$): δ 5.39 (d, 1H, $J = 3.3$ Hz, H-4′), 5.26 (d, 1H, $J = 8.5$ Hz, H-1), 5.17 (dt, 1H, $J = 2.7$, 7.9, 10.6 Hz, H-2′), 4.52 (d, 1H, $J = 8.0$ Hz, H-1′), 3.59 (dd, 1H, $J = 3.4$, 10.6 Hz, H-3′), 3.53 (dd, 1H, $J = 8.1$, 9.7 Hz, H-4), 2.16, 2.15, 2.13, 1.93 (4s, 3H each, Ac); FABMS *m*/*z* calcd for $[C_{30}H_{31}BrCl_4NO_8+Na]^+$: 928.9621. Found: 928.9612.

3.4. 2-Bromoethyl 2,4,6-tri-*O*-acetyl-3-azido-3-deoxy-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside (5)

Compound **4** (110 mg, 0.12 mmol) was dissolved in pyridine (1.0 mL, 0.012 mmol) and Ac_2O (0.60 mL, 6.3 mmol) was added followed by DMAP (catalytic amount). The reaction mixture was stirred overnight at rt under a nitrogen atmosphere. The crude material was co-concentrated with toluene under reduced pressure. The residue was purified by chromatography (3:2, heptane/EtOAc) to give **5** (81 mg, 70%): $[\alpha]_D^{22}$ +0.9 (*c* 0.7, CH_3OH); ^1H NMR (300 MHz, $CDCl_3$): δ 5.67 (dt, 1H, $J = 8.3$, 10.5 Hz, H-3), 5.39 (dd, 1H, $J = 1.0$, 3.2 Hz, H-4′), 5.38 (d, 1H, $J = 8.5$ Hz, H-1), 5.06 (dd, 1H, $J = 7.8$, 10.6 Hz, H-2′), 4.52 (dd, 1H, $J = 2.0$, 12.0 Hz, H-6 or H-6′), 4.51 (d, 1H, $J = 7.8$ Hz, H-1′), 3.53 (dd, 1H, 3.4, 10.6 Hz, H-3′), 3.42–3.30 (m, 2H, CH_2Br), 2.18, 2.17, 2.16, 2.09, 1.97 (5s, 3H each, Ac); FABMS *m*/*z* calcd for $[C_{32}H_{33}BrCl_4N_4O_{16}+Na]^+$: 970.9727. Found: 970.9715.

3.5. 2-Bromoethyl 2,4,6-tri-*O*-acetyl-3-deoxy-3-(2-naphthamido)-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside (6)

HCl (1 M, 0.75 mL, 0.75 mmol) and Pd/C (10%, 65 mg) were added to a solution of **5** (67 mg, 0.070 mmol) in EtOH (50 mL). The mixture was hydrogenated (H_2, 1 atm) for 70 min, filtered through Celite, and concentrated without heating to give the intermediate amine, which was immediately used without further purification. The amine was dissolved in dry CH_2Cl_2 (25 mL), followed by the addition of 2-naphthoyl chloride (98 mg, 0.51 mmol) and pyridine (0.45 mL, 5.6 mmol) under a nitrogen atmosphere. The reaction was monitored with TLC and the reaction mixture was concentrated under reduced pressure when the amine had been consumed. The residue was purified by chromatography (5:4, heptane/EtOAc) to give **6** (43 mg, 56%): $[\alpha]_D^{22}$ +35.1 (*c* 0.7, CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$): δ 8.18 (br s, 1H, Ar-1), 7.95–7.85 (m, 3H, Ar-4, Ar-5, Ar-8), 7.69 (dd, 1H, $J = 1.8$, 8.5 Hz, Ar-3), 7.60–7.52 (m, 2H, Ar-6, Ar-7), 6.60 (d, 1H, $J = 7.6$ Hz, N-H), 5.72 (dd, 1H, $J = 8.8$, 10.5 Hz, H-3), 5.56 (d, 1H, $J = 3.1$ Hz, H-4′), 5.40 (d, 1H, $J = 8.5$ Hz, H-1), 5.02 (dt, 1H, $J = 7.7$, 11.2 Hz, H-2′), 4.70 (d, 1H, $J = 7.8$ Hz, H-1′), 4.62 (dd, 1H, $J = 1.9$, 12.1 Hz, H-6 or H-6′), 4.49 (ddd, 1H, $J = 3.4$, 7.7, 11.1 Hz, H-3′), 3.38 (m, 2H, CH_2Br), 2.17, 2.12, 2.11, 2.07, 1.97 (5s, 3H each, Ac); FABMS *m*/*z* calcd for $[C_{43}H_{41}BrCl_4N_2O_{17}+Na]^+$: 1099.0240. Found: 1099.0230.

3.6. 2-Azidoethyl 2,4,6-tri-*O*-acetyl-3-deoxy-3-(2-naphthamido)-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside (7)

To a mixture of **6** (38 mg, 0.035 mmol) and 15-crown-5 (10 μL, 0.050 mmol) in dry DMF (1 mL) was added NaN_3 (9.5 mg, 0.15 mmol). The mixture was stirred under a nitrogen atmosphere at rt for 24 h. The reaction mixture was concentrated under reduced pressure when **6** had been consumed. The residue was purified by chromatography (1:1, heptane/EtOAc) to give **7** (36 mg, 98%): $[\alpha]_D^{21}$ +35.6 (*c* 0.6, CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$): δ 8.18 (br s, 1H, Ar-1), 7.93–7.84 (m, 3H, Ar-4, Ar-5, Ar-8), 7.68 (dd, 1H, $J = 1.7$, 8.6 Hz, Ar-3), 7.59–7.51 (m, 2H, Ar-6, Ar-7), 6.61 (d, 1H, $J = 7.6$ Hz, N-H), 5.70 (m, 1H, H-3), 5.55 (d, 1H, $J = 3.0$ Hz, H-4′), 5.43 (d, 1H, $J = 8.4$ Hz, H-1), 5.02 (dd, 1H, $J = 7.8$, 11.1 Hz, H-2′), 4.70 (d, 1H, $J = 7.8$ Hz, H-1′), 4.49 (ddd, 1H, $J = 3.1$, 8.6, 11.0 Hz, H-3′), 3.45, 3.14 (2m, 1H each, CH_2N_3), 2.16, 2.10, 2.07, 2.04, 1.96 (5s, 3H each, Ac).

3.7. 2-Azidoethyl 2,4,6-tri-*O*-acetyl-3-deoxy-3-(2-naphthamido)-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (8)

Dry 1,2-diaminoethane (11 μL, 0.16 mmol) was added to a solution of **7** (15 mg, 0.014 mmol) in dry EtOH (1.6 mL). The mixture was kept at 60 °C under a nitrogen atmosphere for 12 h. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in pyridine (0.780 mL) followed by the addition of Ac_2O (0.390 mL) and DMAP (catalytic amount) and stirred under a nitrogen atmosphere at rt overnight. The residue was purified by chromatography (1:1, toluene/EtOH) to give **8** (10 mg, 90%): $[\alpha]_D^{21.5}$ +22.5 (*c* 0.4, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.19 (br s, 1H, Ar-1), 7.94–7.86 (m, 3H, Ar-4, Ar-5, Ar-8), 7.69 (dd, 1H, $J = 1.7$, 8.6 Hz, Ar-3), 7.60–7.52 (m, 2H, Ar-6, Ar-7), 6.58 (d, 1H, $J = 7.6$ Hz, N-H), 5.57 (d, 1H, $J = 2.6$ Hz, H-4′), 5.53 (d, 1H, $J = 9.0$ Hz, N-H), 5.21 (dd, 1H, $J = 8.8$, 10.3 Hz, H-3), 5.02 (dd, 1H, $J = 7.8$, 11.2 Hz, H-2′), 4.68 (d, 1H, $J = 7.8$ Hz, H-1′), 4.64 (d, 1H, $J = 8.1$ Hz, H-1), 4.60 (dd, 1H, $J = 2.3$, 11.9 Hz, H-6 or H-6′), 4.50 (ddd, 1H, $J = 3.4$, 7.7 Hz, H-3′), 4.17 (dd, 1H, $J = 5.0$, 11.9 Hz, H-6 or H-6′), 3.85 (t, 1H, $J = 9.1$ Hz, H-4), 3.50 (ddd, 1H, $J = 3.3$, 8.5, 13.3 Hz, CH_2N_3), 3.26 (ddd, 1H, $J = 3.2$, 7.8, 13.4 Hz, CH_2N_3), 2.14, 2.12, 2.10, 2.08, 1.97 (5s, 3H each, Ac); FABMS *m*/*z* calcd for $[C_{37}H_{45}N_5O_{16}+Na]^+$: 838.2759. Found: 838.2757.

3.8. 2-Azidoethyl 3-deoxy-3-(2-naphthamido)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (9)

Compound **8** (8.7 mg, 0.011 mmol) was dissolved in CH_3OH (4 mL), and 1 M $NaOCH_3$ (40 μL) was added. The reaction was stirred overnight at rt and then neutralized with Duolite C436 (H^+) resin, filtered and concentrated under reduced pressure. The residue was purified by chromatography (6:1, CH_2Cl_2/CH_3OH) to give **9** (4.5 mg, 70%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.47 (br s, 1H, Ar-1), 8.00–7.90 (m, 4H, Ar-3, Ar-4, Ar-5, Ar-8), 7.60–7.53 (m, 2H, Ar-6, Ar-7), 4.58 (d, 1H, $J = 7.7$ Hz, H-1′), 4.53 (d, 1H, $J = 8.2$ Hz, H-1), 4.20 (dd, 1H, $J = 3.3$, 10.9 Hz, H-3′), 1.98 (s, 3H, Ac); FABMS *m*/*z* calcd for $[C_{27}H_{35}N_5O_{11}+Na]^+$: 628.2231. Found: 628.2227.

3.9. 2-(Fluorescein-5/6-amido)ethyl 3-deoxy-3-(2-naphthamido)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (10)

HCl (1 M, 40 μL) and Pd/C (10%, 10 mg) were added to a solution of **9** (4.0 mg, 0.010 mmol) in EtOH (4 mL). The mixture was hydrogenated (H_2, 1 atm) for 100 min, filtered through Celite, and concentrated under reduced pressure without heating to give the crude intermediate amine, which was immediately used without further purification. The crude amine was dissolved in base (0.1 M $NaHCO_3/H_2O$, 0.5 mL). NHS-fluorescein (13.9 mg, 0.029 mmol) was dissolved in DMSO (0.5 mL) and added to the reaction mixture. The flask was covered in Al-foil and stirred at rt for 150 min. The reaction mixture was partially purified through Sephadex LH20 (1:1, CH_3OH/CH_2Cl_2), concentrated, dissolved in H_2O and applied to C18 silica (0.5 g). Excess reagents and impurities were washed away with H_2O, followed by elution with 25% and 50% CH_3OH to give **10** (1.3 mg, 21%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.57 (m, fluorescein), 7.99 (br s, 1H, Ar-1), 8.02–7.90 (m, 4H, Ar-3, Ar-4, Ar-5, Ar-8), 7.95 (br s, fluorescein), 7.62–7.55 (m, 2H, Ar-6, Ar-7), 7.35 (d, fluorescein), 7.05 (m, fluorescein), 6.55 (m, fluorescein), 4.58 (d, 1H, $J = 7.35$ Hz, H-1′), 4.52 (d, 1H, $J = 7.81$ Hz, H-1), 4.02 (d, 1H, $J = 2.3$ Hz, H-4′), 1.91 (s, 3H, Ac); MALDIMS calcd for $[C_{48}H_{47}N_3O_{17}+Na]^+$: 960.3. Found: 960.3.

3.10. Fluorescence polarization experiments

Fluorescence polarization experiments were performed as generally described in Ref. 18 and detailed in Ref. 24. Fluorescence polarization experiments were performed at 4 °C. Galectin-3 was also evaluated at ambient temperature. Dissociation constants, maximum anisotropy values, and standard errors were obtained by plotting anisotropy values against the galectin concentrations and fitted to the binding isotherm $A = A_0 + (A_{max} - A_0) * ([G]/(K_d + [G]))$[18] using a nonlinear Levenberg–Marquart algorithm implemented in KaleidaGraph 4.0 (Synergy Software 2005).

3.11. Molecular dynamics simulations

Molecular dynamics simulations (100 ns) of the methyl glycoside analogs of **10** and **11** in complex with galectin-3 were performed with the OPLS2001 force field[25] in water implemented in Macromodel 8.5. The starting structure for the analog of **10** was built from the crystal structure of galectin-3 in complex with methyl 3-deoxy-3-(4-methoxy-2,3,5,6-tetrafluorobenzamido)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-4-*O*-β-D-glucopyranoside.[20] The crystal structure of the analog of **11** in complex with galectin-3[20] was used as the starting structure for **11**. Overlay of the sampled conformers of **10** and **11** (Fig. 2) was done based on three of the most central and rigid amino acid residues; ala156, asn160, and gly235.

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Supplementary data

^{1}H NMR spectra of **2** and **4**–**10**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.04.027.

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