Low Micromolar Inhibitors of Galectin-3 Based on 3′-Derivatization of *N*-Acetyllactosamine

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*A strategy for generating potential galectin inhibitors was devised based on derivatization at the C-3′ atom in 3′-amino-*N*-acetyllactosamine by using structural knowledge of the galectin carbohydrate recognition site. A collection of 12 compounds was prepared by* N*-acylations or* N*-sulfonylations. Hydrophobic tagging of the O-3 atom in the* N*-acetylglucosamine residue with a stearic ester allowed rapid and simple product purification. The compounds were screened in a galectin-3 binding assay and three compounds with significantly higher inhibitory activities compared to the parent* N*-acetyllactosaminide were found. These three best inhibitors all carried an aromatic amide at the C-3′ position of the galactose moiety, which indicates that favorable interactions were formed between the aromatic group and galectin-3. The best inhibitor had an IC_{50} value (4.4 μM) about 50 times better than the parent* N*-acetyllactosaminide, which implies that it has potential as a valuable tool for studying galectin-3 biological functions and also as a lead compound for the development of galectin-3-blocking pharmaceuticals.*

KEYWORDS:

carbohydrates · galectin-3 · inhibitors · *N*-acetyllactosamine · parallel synthesis

Introduction

Galectin-3 is a member of a family of small cytosolic proteins defined by their affinity for β-galactosides and characteristic amino acid sequence motifs. Twelve members of this family which are found in mammals have been reported so far.[1] These proteins typically bind extracellular carbohydrates and are secreted by nonclassical pathways.[2] Therefore, most attention has been given to their possible extracellular actions, such as binding and cross linking glycoconjugate ligands, possibly forming supramolecular arrays[3] to modulate cell adhesion and cell signaling.[4] Galectins, as cytosolic and nuclear proteins, have also been proposed to modulate intracellular processes such as RNA splicing, apoptosis, and the cell cycle.[1c, 4]

There is strong evidence to suggest a role for galectins, in particular galectin-3, in immunity regulation, inflammation, and cancer, although their precise mechanisms of action remain unclear. A proinflammatory role for galectin-3 is indicated by its induction in inflammatory conditions, a large variety of effects on immune cells (for example, oxidative burst in neutrophils, chemotaxis in monocytes), and alterations of the inflammatory response (mainly in the neutrophils) in null mutant mice.[4, 5] Recently, galectin-3 has also been implicated as a regulator in the formation of the "immunological synapse" between T-cells and antigen-presenting cells.[6] Correlative and experimental evidence in various systems suggests a cancer-promoting effect of galectin-3 and it has been proposed to have an anti-apoptotic action, to promote angiogenesis, or to promote metastasis by affecting cell adhesion.[4, 7] Glycoconjugates which decrease metastasis in mice have been suggested, but not proven, to act by inhibition of galectins.[8]

Potent specific inhibitors of galectins are highly desirable as basic research tools to elucidate galectin functions and as lead compounds for novel anti-inflammatory and anticancer agents since galectin-3 may be involved in these pathological conditions.[4, 5] Easily available known saccharide ligands (lactose, *N*-acetyllactosamine, and thiodigalactosides) have low inhibitory potency, with concentrations in the 0.2 – 1 mM range needed for 50% inhibition ($IC_{50} = 0.2 - 1$ mM). Concentrations of these compounds over 10 mM are usually needed for effective inhibition of galectin activity in biological systems.[4, 9] Certain aminoglycosides with anticancer activity also show a similarly low affinity for

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galectins.[8b] Some naturally occurring and/or synthetic saccharides show IC_{50} values in the 10 – 30 μM range.[4, 9, 10] Oligovalent lactose derivatives may show strongly improved IC_{50} values compared to free lactose when the IC_{50} is calculated per intact oligovalent molecule, but the improvement is much smaller when calculated per lactose residue.[11, 12]

Herein we report a strategy for designing and synthesizing potent monovalent inhibitors of galectins. This is possible because galectins have a relatively large carbohydrate binding site that can accommodate a molecule as large as a tetrasaccharide, as indicated by the saccharide binding specificities of galectins,[9a–c] modeling,[9d] and galectin structure.[13] Within this site all galectins have a conserved core binding site for lactose and related β-galactoside-containing disaccharides, as shown for galectin-3 in Figure 1. The galactose (Gal) residue is the most

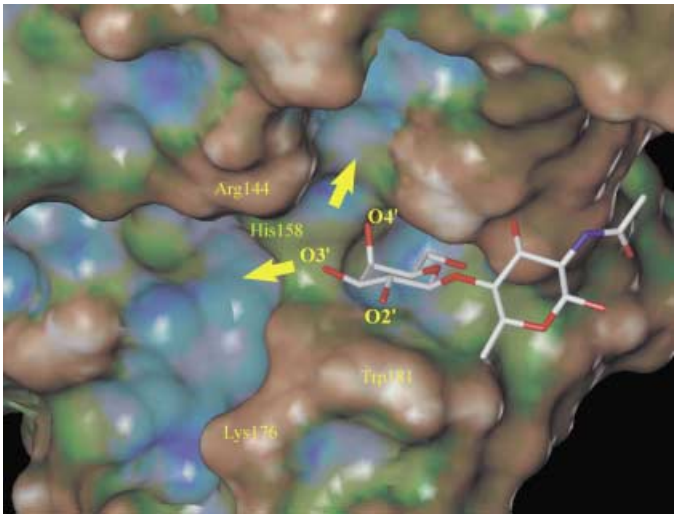
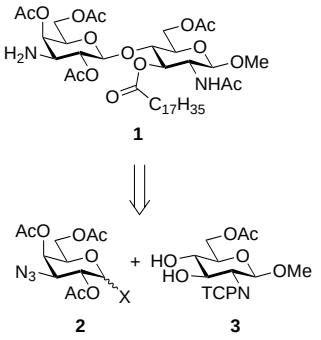


Figure 1. *Connolly surface of the galectin-3 carbohydrate recognition domain with the ligand* N*-acetyllactosamine, based on the X-ray crystal structure of the complex.[13a] The picture indicates the possibility of an extended binding site close to O-3′ (see arrows). The colour coding of the site indicates curvature, with blue for concave and brown for convex surface. The surface was created with the MOLCAD module of the SYBYL program (Tripos Inc., St. Louis).*

deeply buried part of the disaccharide. The HO-4′ (′ indicates a position in the Gal unit rather than the glucosamine), and HO-6′ groups hydrogen-bond to the protein and the H-3′, H-4′, and H-5′ atoms together form a hydrophobic patch that makes van der Waals contact with a tryptophan side-chain. This leaves only the HO-3′ and HO-2′ groups available for further extensions. The HO-3′ group is pointing directly into an extended groove of the protein (Figure 1). Thus, derivatization of this position with diverse structural extensions was an attractive strategy for creation of additional favorable interactions with the protein and, thereby, for discovery of high-affinity inhibitors for galectin-3. Further support for this idea is given by certain natural saccharides extended at this position which have a strongly enhanced affinity for galectin-3.[9]

An *N*-acetyllactosamine derivative **1** (Scheme 1), in which the HO-3′ group was replaced by an amino group, was chosen as a starting material for diversification at the C-3′ atom. The amino



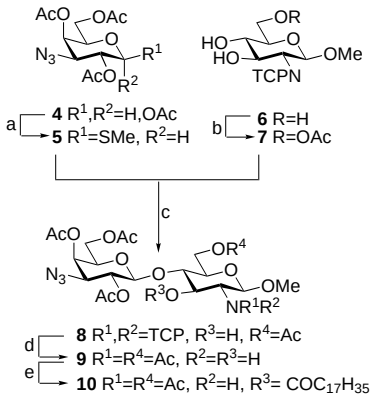
Scheme 1. *Retrosynthetic analysis of the 3′-amino-*N*-acetyllactosamine derivative* ***1*** *which is amenable to parallel solution synthesis of galectin-3 inhibitors.*

group is a versatile handle for the introduction of a wide variety of functional groups under mild and simple conditions. Henceforth, we could exploit acylation and sulfonylation reactions with a large number of commercially available reagents.

Retrosynthetic analysis (Scheme 1) suggested that an *N*-acetyllactosamine derivative such as **1** should be accessible through glycosylation of a 6-*O*-acyl-protected *N*-tetrachlorophthalimido (*N*-TCP) glucosamine derivative **3** with a 3-azidogalactosyl donor **2**. Compound **2** is accessible from D-glucose by a known route[14] and 6-*O*-protected *N*-TCP glucosamine derivatives are regioselectively galactosylated at the HO-4 group.[15] In addition, the *N*-acetyllactosamine derivative **1** was equipped with a stearoyl ester, at the C-3 atom of the *N*-acetylglucosamine (GlcNAc) to act as a hydrophobic tag and permit facile product purification by reversed solid-phase extraction.[16–18]

Results and Discussion

The 3-azido-galactosyl donor, thiogalactoside (**5**), was prepared in 86% yield by treating the known 1-*O*-acetate **4**[14] with trimethylsilyl trifluoromethanesulfonate (TMSOTf) and (methylthio)trimethylsilane ($MeSSiMe_3$) in $(CH_2Cl)_2$ and stirring for seven days (Scheme 2). The thiogalactoside **5** was preferable to the known corresponding galactosyl bromide as a donor[14] because



Scheme 2. *a) $MeSSiMe_3$, TMSOTf, $(CH_2Cl)_2$, 7 days, 86%; b) AcCl, sym-collidine, CH_2Cl_2, −42 °C, 7 h, 75%; c) NIS, TfOH, CH_2Cl_2, AW-300 molecular sieves, −42 °C, 2 h, 75%; d) 1. $H_2N(CH_2)_2NH_2$, EtOH, 60 °C, 7.5 h; 2. MeOH, H_2O, Ac_2O, 12 h, 83%; e) $C_{17}H_{36}COCl$, DMAP, pyridine, CH_2Cl_2, 24 h, 80%. For abbreviations, see text.*

5 could be obtained in a higher yield than the bromide from **4** and because **5** is more stable upon storage.

It is well known that 6-*O*-benzyl-protected *N*-TCP glucosamine derivatives are regioselectively galactosylated at the HO-4 group in excellent yields.[15] However, a 6-*O*-acyl protection group was desirable, because it shortened the final deprotection procedure to one step and avoided potential regioselectivity problems upon hydrogenation of the azido functionality. Fortunately, the 6-*O*-acetyl-protected *N*-TCP glucosamine derivative **7** could be obtained in 75% yield by regioselective acetylation of **6** with acetyl chloride and sym-collidine buffer in CH_2Cl_2 (Scheme 2).

Glycosylation of the acceptor **7** with the thiogalactoside **5** was attempted in CH_2Cl_2 with MeSBr/AgOTf, Br_2/AgOTf, or *N*-iodosuccinimide (NIS)/TfOH as a promoter. The promoter NIS/TfOH provided the best yield and regioselectivity, and compound **8** was obtained in 75% yield (Scheme 2). The *N*-TCP functionality was converted into the *N*-acetate **9** in 83% yield by using standard conditions.[19–21] The regioselectivity of the glycosylation reaction was verified by acetylation of the HO-3 group of **9**, which resulted in a downfield shift of 1.1 ppm in the ^{1}H NMR spectrum for the H-3 atom. Treatment of **9** with steaoryl chloride, pyridine, and 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 gave compound **10** tagged with a hydrophobic stearic ester to allow convenient and rapid product purification during the parallel synthesis by means of reversed solid-phase extraction described below.

Reduction of the azido group in compound **10** was accomplished by catalytic hydrogenation in ethanol/HCl over Pd/C to give the corresponding amine **11**, which served as the starting material for the *N*-derivatization reactions (Table 1). However, **11** was found to be highly prone to acetyl migration to the amine from neighboring *O*-acetates, which interfered with the subsequent acylations/sulfonylations of **11** and resulted in disappointingly low yields for the first compounds prepared (**15**, **16**, **19**–**21**, and **23**; Table 1). To minimize the acetyl migration the time allowed for the hydrogenation of **10** was limited to a maximum of 20 minutes, heating of the amine **11** at any time during the reaction and work-up procedure was avoided, and the acylations/sulfonylations were begun within 1 hour of production of **11**. These precautions allowed *N*-acylation and deprotection reactions to proceed in overall yields exceeding 50% for the remaining compounds **13**–**14**, **17**, **18**, and **22** (Table 1).

Each *N*-acylation product was purified by C18 solid-phase extraction.[16] The *O*-acetyl and stearoyl groups were then removed by methanolysis, followed by C18 solid-phase extraction to yield the final products (**13**–**23**). Upon the deprotection of the precursor to compound **18** with methanolic sodium methoxide, the pentafluorobenzamide moiety underwent a nucleophilic aromatic substitution to give the corresponding *p*-methoxy-substituted benzamide **18**. Compounds **22**–**23** carrying *tert*-butoxycarbonyl-protected (Boc-protected) amines were treated with trifluoroacetic acid (TFA)/CH_2Cl_2 before acyl group removal with methanolic sodium methoxide. The compounds were 80–98% pure as determined by NMR spectroscopy.

A competitive enzyme-linked immunosorbent assay (ELISA) was used to evaluate compounds **13**–**23** as galectin-3 inhibitors. In this ELISA, the binding of a Galα1-3′LacNAc-horseradish peroxidase conjugate to galectin-3-coated microwells was measured and the percentage decrease in this binding in the presence of the test compound recorded as inhibitory potency. First, the compounds were screened at a concentration of 0.04 mM and compared to the underivatized parent compound, the methyl glycoside of *N*-acetyllactosamine **24** (Figure 2). Some compounds were equal to or worse than **24** as inhibitors of galectin-3. Interestingly, the unmodified 3′-amino-derivative **12** was significantly poorer than **24** as an inhibitor.

Seven compounds were significantly better than **24**. The aromatic amides (**16**–**18**) provided the largest improvement in inhibitory activity and were analyzed further (Figure 3). The best

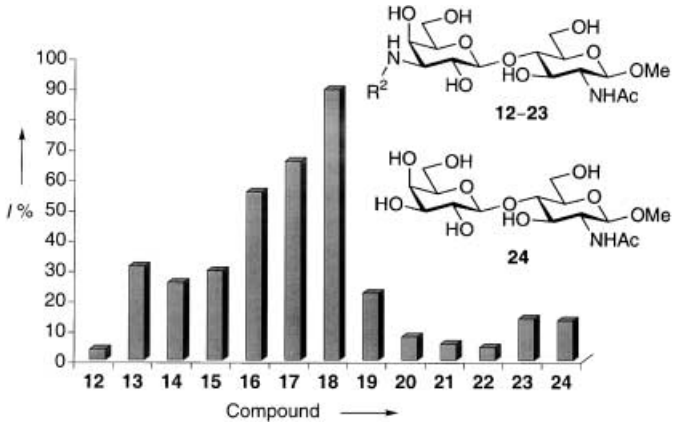


Figure 2. *Screening of the compounds **12**–**24** for inhibition of galectin-3 (see Table 1 for R^2). The ability of compounds **12**–**24** (each tested at final concentrations of 0.04 mM) to inhibit the binding of Galα3Galβ4GlcNAcβ-HRP to immobilized galectin-3 in microtiter plates is shown as % inhibition. I = inhibition.*

Inhibitor no.	13	16	17	18	24
Symbol	□	△	▽	●	■
Est. IC_{50} [μM]	118	14	13	4.4	207

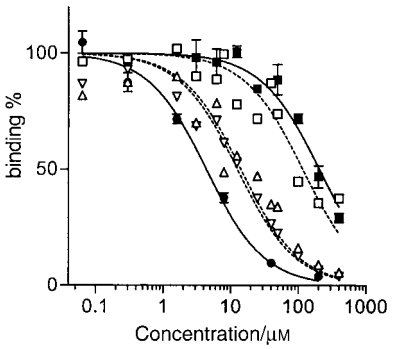
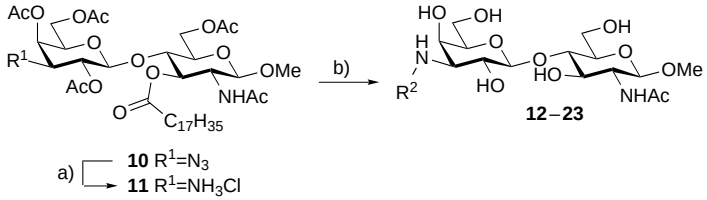
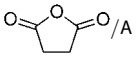
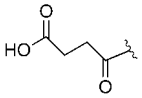
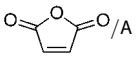
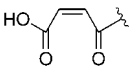
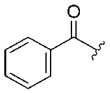
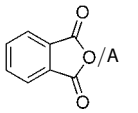
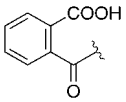
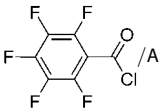
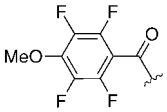
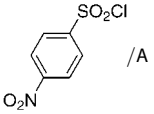
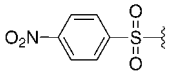
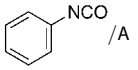
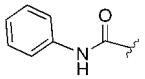
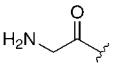
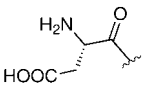


Figure 3. *Titration of the four best inhibitors found amongst compounds **12**–**24**. The inhibitory ability of a series of concentrations of the four best inhibitors **13** and **16**–**18**, as well as the parent compound methyl N-acetyl-β-lactosaminide **24**, were examined in the same assay as used in Figure 2. The data were fit by nonlinear regression to the formula for single site competitive inhibition: $Y = 100/1 + 10^{(X - \log(IC_{50}))}$, where Y = binding with the inhibitor as a percentage of binding without the inhibitor and X = logarithm of the inhibitor concentration in μM. The best inhibitor (**18**) and the reference compound (**24**) are shown by closed symbols and solid lines (with error bars showing the standard error in the mean, based on 4 or 6 measurements, respectively). The other compounds are shown by open symbols (without error bars) and dotted lines.*

Table 1. *Synthesis and spectroscopic data of 3′-amino-*N*-acetyllactosamine derivatives* ***12*** *–* ***23****.*



	Reagents/ conditions[a]	$R^2=$	Yield [%]	^{1}H NMR data (400 MHz, D_2O) δ	HRMS[c] calcd/found
12	[24]	H	59	4.59 (d, 1H, $J=7.6$ Hz, H-1′) 4.49(d, 1H, $J=7.1$ Hz, H-1)	397.1822/397.1824
13	Ac_2O/A	Ac	79	4.58 (d, 1H, $J=7.8$ Hz, H-1′) 4.48 (d, 1H, $J=7.7$ Hz, H-1) 2.07, 2.05 (2s, 3H each, Ac)	461.1747/461.1750
14			52	4.58 (d, 1H, $J=7.8$ Hz, H-1′) 4.48 (d, 1H, $J=7.9$ Hz, H-1) 2.55 (m, 2H, $COCH_2$) 2.48 (m, 2H, $COCH_2$)	519.1802/519.1802
15			32	6.37, 6.12 (2d, 1H each, $J=12.3$ Hz, =CH) 4.59 (d, 1H, $J=7.7$ Hz, H-1′) 4.47(d, 1H, $J=7.7$ Hz, H-1)	517.1646/517.1659
16	BzCl/A		24	7.69 (m, 5H, Ar) 4.65 (d, 1H, $J=7.7$ Hz, H-1′) 4.49 (d, 1H, $J=7.9$ Hz, H-1)	523.1904/523.1909
17			70	7.59 (m, 4H, Ar) 4.65 (d, 1H, $J=7.8$ Hz, H-1′) 4.48 (d, 1H, $J=8.1$ Hz, H-1)	567.1802/567.1802
18			81	4.64 (d, 1H, $J=7.9$ Hz, H-1′) 4.49 (d, 1H, $J=7.9$ Hz, H-1) 4.17 (s, 3H, OMe)	625.1633/625.1652
19	CH_3SO_2Cl/A	CH_3SO_2	10	4.55 (d, 1H, $J=6.8$ Hz, H-1′) 4.47 (d, 1H, $J=8.0$ Hz, H-1) 3.16 (s, 3H, Me)	497.1417/497.1415
20			7	8.42, 8.13 (2d, 2H each, $J=8.9$ Hz, Ar) 4.45 (d, 1H, $J=7.8$ Hz, H-1) 4.44 (d, 1H, $J=7.2$ Hz, H-1′)	604.142/604.326[d]
21			15	7.71 (m, 5H, Ar) 4.61 (d, 1H, $J=7.8$ Hz, H-1′) 4.48 (d, 1H, $J=7.8$ Hz, H-1)	538.2013/538.2022
22	Boc-Gly-OH/B		68	4.59 (d, 1H, $J=7.4$ Hz, H-1′) 4.47 (d, 1H, $J=8.1$ Hz, H-1)	476.1856/476.1855
23	Boc-Asp(O*t*Bu)-OH/B		9	4.59 (d, 1H, $J=7.5$ Hz, H-1) 4.48 (d, 1H, $J=8.4$ Hz, H-1′)	534.191/534.543[d]

[a] 10% Pd/C, H_2 (1 atm.), HCl, EtOH, 20 min. [b] A: 1. pyridine, CH_2Cl_2; 2. NaOMe/MeOH; B: 1. DIC, CH_2Cl_2; 2. TFA, CH_2Cl_2; 3.NaOMe/MeOH. DIC = diisopropylcarbodiimide. [c] $[M+Na]^+$. [d] MALDI-TOF MS.

inhibitor, **18**, had an IC_{50} value of 4.4 μM averaged over three experiments. The extended binding site (Figure 1) is lined with the amino acids Arg 144, His 158, and Lys 176 and it is tempting to speculate that the enhanced affinity of **16** – **18** is a result of interaction with these amino acids. Model studies suggest that these amino acids interact with extended natural oligosaccharides.[9d] However, further structural studies on the inhibitor – galectin-3 complexes (NMR and/or x-ray) are necessary for rationalization of the enhanced inhibitory potency of **16** – **18**.

In conclusion, we have shown that our inhibitor design strategy is valid and a 3′-amino analogue of *N*-acetyllactosamine provides a favorable scaffold for parallel synthesis of galectin-3 inhibitors. The best inhibitor **18** was about 50 times as potent as the parent *N*-acetyllactosamine derivative **24**, which is better than any monovalent galectin inhibitor known to date. This

compound and future improved variants should be valuable tools for evaluating the functions of galectin-3 in a biological system and promising lead candidates for the development of galectin-3-blocking pharmaceuticals.

Experimental Section

General methods and materials: NMR spectra were recorded with a Bruker DRX-400 instrument. Chemical shifts are given in ppm, with reference to internal $CHCl_3$ ($\delta = 7.26$) or HDO ($\delta = 4.81$). Chemical shifts and coupling constants were obtained from ^{1}H NMR spectra and proton resonances were assigned from COSY experiments. High-resolution fast atom bombardment mass spectra (HRMS) were recorded with a JEOL SX-120 instrument. Matrix-assisted laser desorption/ionization-time of flight mass spectroscopy (MALDI-TOF MS) was carried out with a Bruker Biflex III instrument. Column chromatography was performed on SiO_2 (Matrex, 60 Å, 35 – 70 μm, Grace Amicon) and thin layer chromatography (TLC) was carried out on 60 F_{254} silica (Merck), developed with aqueous sulfuric acid, and detected under UV light. Solutions were concentrated by using rotary evaporation with a bath temperature at or below 40 °C. CH_2Cl_2 was dried by distillation over CaH_2. Pyridine was dried over 4 Å molecular sieves. MeOH and EtOH were dried over 3 Å molecular sieves. Microwell plates were from Nalge Nunc International (Nunc immuno plate, Maxisorp surface). Recombinant human galectin-3 was produced in *Escherichia coli* and purified as previously described.[22] The Galα3Galβ4GlcNAcβ-horse radish peroxidase conjugate (HRP-2) was from Glycorex AB, Lund, Sweden. Microwell plates were developed with a 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate kit (BioRad 172-1066) according to the manufacturers recommendations.

Methyl 3-azido-3-deoxy-2,4,6-tri-*O*-acetyl-1-thio-β-D-galactopyranoside (5): TMSOTf (0.102 mL, 0.564 mmol) was added to a solution of **4**[14] (231 mg, 0.619 mmol), $MeSSiMe_3$ (0.250 mL, 1.76 mmol), and molecular sieves AW-300 (0.46 g) in 1,2-dichloroethane (3.0 mL) under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 7 days, aqueous Na_2CO_3 (5%, 5 mL) was added, and the mixture was stirred for another 2 hours. The organic layer was separated, washed with water, dried over Na_2SO_4, filtered, and concentrated. The residue was chromatographed (heptane/ethylacetate (2:1)) to give **5** (192 mg, 86%). $[\alpha]_D^{25} = -34.8$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.45$ (dd, 1H, $J = 3.4$, 1.2 Hz, H-4), 5.22 (t, 1H, $J = 10.0$ Hz, H-2), 4.36 (d, 1H, $J = 9.8$ Hz, H-1), 4.15 – 4.07 (m, 2H, H-6, 6′), 3.91 (dt, 1H, $J = 6.6$, 1.2 Hz, H-5), 3.66 (dd, 1H, $J = 10.2$, 3.4 Hz, H-3), 2.19, 2.17, 2.15 (3s, 3H each, Ac), 2.06 (s, 3H, Me); HRMS: calcd for $C_{13}H_{19}N_3NaO_7S$ (M+Na): 384.0841; found: 384.0837.

Methyl 6-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside (7): Acetyl chloride (0.115 mL, 1.62 mmol) was added dropwise to compound **6**[23] (653 mg, 1.42 mmol) and sym-collidine (0.940 mL, 7.09 mmol) in CH_2Cl_2 (25 mL) under a nitrogen atmosphere at −42 °C. The reaction was continued at −20 °C for 4 hours, then additional acetyl chloride (0.025 mL, 0.352 mmol) and sym-collidine (0.400 mL, 3.0 mmol) were added. The reaction was quenched with MeOH (8 mL) after 3 more hours. The reaction mixture was partitioned between CH_2Cl_2 and aqueous HCl (0.5 M). The organic layer was neutralized with aqueous saturated $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was chromatographed (heptane/ethylacetate (1:1)) to give **7** (535 mg, 75%). $[\alpha]_D^{25} = -18.4$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.04$ (d, 1H, $J = 8.5$ Hz, H-1), 4.45 (dd, 1H, $J = 11.9$, 2.2 Hz, H-6), 4.27 (dd, 1H, $J = 11.9$, 5.5 Hz, H-6′), 4.19 (dd, 1H, $J = 10.7$, 8.7 Hz, H-3), 3.93 (dd, 1H, $J = 10.7$, 8.5 Hz, H-2), 3.63 – 3.58 (m, 1H, H-5), 3.44 – 3.41 (m, 1H, H-4), 3.39 (s, 3H, OMe), 2.09 (s, 3H, Ac); HRMS: calcd for $C_{17}H_{15}Cl_4NNaO_8$ (M+Na): 523.9449; found: 523.9447.

Methyl 4-*O*-(2,4,6-tri-*O*-acetyl-3-azido-3-deoxy-β-D-galactopyranosyl)-6-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranoside (8): Compounds **5** (66.1 mg, 0.183 mmol) and **7** (76.9 mg, 0.153 mmol) and activated AW-300 molecular sieves (0.35 g) were stirred in dry CH_2Cl_2 (5.0 mL) for 30 minutes under a nitrogen atmosphere. The mixture was cooled to −42 °C and *N*-iodosuccinimide (51.2 mg, 0.228 mmol) was added, followed by trifluoromethanesulfonic acid (2.0 μL, 22.6 μmol). The reaction mixture was allowed to warm to room temperature after 2 hours, 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. The residue was chromatographed (heptane/ethylacetate (2:1)) to give **8** (93.9 mg, 75%). $[\alpha]_D^{25} = +7.6$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.36$ (d, 1H, $J = 3.1$ Hz, H-4′), 5.15 (q, 1H, $J = 10.6$, 7.9 Hz, H-2′), 5.10 (d, 1H, $J = 8.5$ Hz, H-1), 4.52 (d, 1H, $J = 7.9$ Hz, H-1′), 4.33 (dd, 1H, $J = 11.8$, 1.8 Hz, H-6), 4.28 (m, 2H, H-3, OH), 4.06 – 4.14 (m, 3H, H-6, 2′, 6′), 3.88 – 3.99 (m, 2H, H-5′, 6′), 3.70 (m, 1H, H-5), 3.59 (dd, 1H, $J = 10.6$, 3.4 Hz, H-3′), 3.52 (dd, 1H, $J = 9.6$, 8.2 Hz, H-4), 3.42 (s, 3H, OMe), 2.15, 2.13, 2.12, 1.90 (4s, 3H each, Ac); HRMS: calcd for $C_{29}H_{30}Cl_4N_4NaO_{15}$ (M+Na): 837.0359; found: 837.0374.

Methyl (2,4,6-tri-*O*-acetyl-3-azido-3-deoxy-β-D-galactopyranosyl)-2-acetamido-6-*O*-acetyl-2-deoxy-β-D-glucopyranoside (9): Dry 1,2-diaminoethane (18 μL) was added to a solution of **8** (133 mg, 0.152 mmol) in dry EtOH (13 mL). The mixture was heated at 60 °C for 7.5 hours then co-concentrated with toluene (5 mL). The residue was dissolved in MeOH (15 mL), H_2O (3 mL), and Ac_2O (4.5 mL), stirred overnight, then co-concentrated with toluene (20 mL). The residue was chromatographed (toluene/acetone (1:1)) to give **9** (70.6 mg, 83%). $[\alpha]_D^{25} = +1.6$ ($c = 0.03$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.63$ (d, 1H, $J = 7.8$ Hz, NH), 5.40 (d, 1H, $J = 3.3$ Hz, H-4′), 5.17 (dd, 1H, $J = 10.6$, 8.0 Hz, H-2′), 4.62 (d, 1H, $J = 8.3$ Hz, H-1), 4.54 (d, 1H, $J = 8.0$ Hz, H-1′), 4.34 – 4.31 (m, 2H, OH, H-6), 4.18 (dd, 1H, $J = 10.7$, 3.7 Hz, H-6′), 4.09 – 3.93 (m, 4H, H-6, 5′, 3, 6′), 3.62 – 3.57 (m, 2H, H-5, 3′), 3.48 (s, 3H, OMe), 3.51 – 3.44 (m, 2H, H-4,2), 2.17, 2.16, 2.11, 2.06, 2.01 (5s, 3H each, Ac); HRMS: calcd for $C_{23}H_{34}N_4NaO_{14}$ (M+Na): 613.1969; found: 613.1972.

Methyl 4-*O*-(2,4,6-tri-*O*-acetyl-3-azido-3-deoxy-β-D-galactopyranosyl)-2-acetamido-6-*O*-acetyl-2-deoxy-3-*O*-stearoyl-β-D-glucopyranoside (10): Stearoyl chloride (0.160 mL, 0.475 mmol) was added to a solution of **9** (65.9 mg, 0.112 mmol), pyridine (0.45 mL), and DMAP (catalytic amount) in dry CH_2Cl_2 (10 mL) under a nitrogen atmosphere, at −78 °C. The mixture was allowed to warm to room temperature, then quenched with EtOH (2 mL) after 24 hours, and concentrated. The residue was chromatographed (toluene/acetone (3:1)) to give **10** (75.8 mg, 79%). $[\alpha]_D^{25} = -16.4$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.72$ (d, 1H, $J = 9.5$ Hz, NH), 5.39 (d, 1H, $J = 3.3$ Hz, H-4′), 5.07 – 5.02 (m, 2H, H-2′,3), 4.46 (dd, 1H, $J = 11.9$, 8.9 Hz, H-6), 4.44 (d, 1H, $J = 7.9$ Hz, H-1′), 4.33 (d, 1H, $J = 7.2$ Hz, H-1), 4.19 (dd, 1H, $J = 11.9$, 5.4 Hz, H-6), 4.08 – 4.03 (m, 3H, H-2, 6′, 6′), 3.85 – 3.82 (m, 1H, H-5′), 3.74 (t, 1H, $J = 8.1$ Hz, H-4), 3.66 – 3.61 (m, 1H, H-5′), 3.58 (dd, 1H, $J = 10.6$, 3.4 Hz, H-3′), 3.44 (s, 3H, OMe), 2.28 (t, 2H, $J = 7.6$ Hz, $COCH_2$), 2.14, 2.12, 2.11, 2.06, 1.95 (5s, 3H, Ac), 1.51 – 1.64 (m, 2H, $COCH_2CH_2$), 1.23 (br s, 28H, CH_2), 0.88 – 0.85 (m, 3H, CH_3); HRMS: calcd for $C_{41}H_{68}N_4NaO_{15}$ (M+Na): 879.4579; found: 879.4596.

Typical procedure for acylations and sulfonylations (synthesis of compounds 13 – 21): 1 M HCl (0.34 mL, 0.34 mmol) and Pd/C (10%, 33.5 mg) were added to a solution of **10** (29.0 mg, 38.8 μmol) in EtOH (degassed, 20 mL). The mixture was hydrogenated (H_2, 1 atm) for

20 minutes, filtered through Celite, and concentrated without heating to give the crude intermediate amine **11**, which was immediately used without further purification. The crude **11** was dissolved in dry CH_2Cl_2 (10 mL), then pentafluorobenzoyl chloride (49 μL, 0.34 mmol) and pyridine (15 μL, 0.19 mmol) were added under a nitrogen atmosphere. The reaction was monitored by TLC and the reaction mixture was concentrated when **11** had been consumed. The residue was dissolved in 70% MeOH and applied to C18 silica (3 g). Excess reagents and impurities were washed away with 70% MeOH, after which elution with 100% MeOH gave a protected intermediate (31.2 mg, 90%) after concentration. The residue was dissolved in MeOH (4.0 mL), and 1 M NaOMe (0.6 mL) was added. The reaction was continued overnight and then neutralized with Duolite C436 (H^+) resin, filtered, and concentrated. The residue was dissolved in water and applied to C18 silica (3 g). Excess reagents and impurities were washed away with water, after which elution with 30% MeOH gave **18** (16.5 mg, 92%).

Typical procedure for acylation with amino acids (synthesis of compounds 22 and 23): 1 M HCl (0.135 mL, 0.135 mmol) and Pd/C (10%, 12.0 mg) were added to a solution of **10** (11.3 mg, 13.2 μmol) in EtOH (degassed, 20 mL). The mixture was hydrogenated (H_2, 1 atm) for 20 minutes, filtered through Celite, and concentrated without heating to give the crude intermediate amine **11**, which was immediately used without further purification. A solution of *N*-Boc-glycine (9.0 mg, 51.4 μmol) in dry CH_2Cl_2 (8 mL) was added to the crude **11** under nitrogen atmosphere, followed by *N*,*N*′-diisopropylcarbodiimide (10 μL, 64.6 μmol) and pyridine (15 μL, 0.19 mmol). The reaction was kept at room temperature overnight then co-concentrated with toluene under reduced pressure. The residue was dissolved in 70% MeOH and applied to C18 silica (3 g). Excess reagents and impurities were washed away with 70% MeOH and elution with 100% MeOH gave a protected intermediate (13.1 mg, quantitative) after concentration. TFA (0.5 mL) was added to the residue in dry CH_2Cl_2 (5.0 mL). The reaction mixture was co-concentrated with toluene (15 mL) after 5 hours and the residue was purified by C18 solid-phase extraction as described above. The residue was dissolved in MeOH (4.0 mL), NaOMe (0.6 mL, 1 M) was added, and then the reaction was left overnight, neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated. The residue was dissolved in water and applied onto C18 silica (3 g). Elution with water gave **22** (4.1 mg, 84%).

Screening ELISA: Microtiter plates were coated with recombinant galectin-3 (10 μg mL^{-1}, 50 μL/well) from *E. coli* at 4 °C overnight, then washed three times with phospate-buffered saline containing 0.05% Tween 20 (PBS-T). The wells were blocked with PBS-T containing 1% BSA (PBSA-T; 100 μL/well) for 1 hour at room temperature, followed by washing with PBS-T. Compounds **12** – **24** (100 μL/well, 0.04 mM in PBS-T) were added in duplicate to the wells, followed by Galα3-Galβ4GlcNAcβ-HRP conjugate (100 μL/well, 1 mg mL^{-1} in PBSA-T). The wells were washed with PBS-T after 1 hour incubation at room temperature, followed by development with the TMB-peroxidase substrate kit. The reaction was stopped after 60 min by addition of 1 N sulfuric acid (100 μL/well), and the optical density was read at 450 nm. Each experiment was conducted twice with each sample in duplicate. The pH values of all the compound stock solutions were checked before testing and were all shown to be 7.1.

Titration ELISA: Microtiter plate wells were coated with galectin-3 and blocked as described above. 125 μL of inhibitors **13**, **16** – **18**, and **24** (0.2 mM in PBS-T) was added to the first well. A five fold serial dilution was performed by transferring 25 μL from the first well to a second well containing 100 μL PBS-T, mixing, then transferring 25 μL from the second well to a third well also containing 100 μL PBS-T, and so on to the eighth well from which 25 μL were discarded. The dilution series was done in duplicate. PBS-T (100 μL) only was added to one column of wells (to give the optical density in the absence of inhibitor). PBS-T was also added to one column of wells not coated with galectin-3 (to give the background signal). To each well Galα3Galβ4GlcNAcβ-HRP conjugate (100 μL/well, 1 mg mL^{-1} in PBS-T) was then added. Incubation, washing, and detection were performed as described above. The data was analyzed with nonlinear regression analysis by using the GraphPad Prism 3.00 program for Windows (GraphPad Software, San Diego, CA).

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