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Screening for Galectin-3 Inhibitors from Synthetic Lacto-*N*-biose Libraries Using Microscale Affinity Chromatography Coupled to Mass Spectrometry

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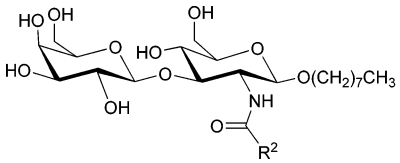
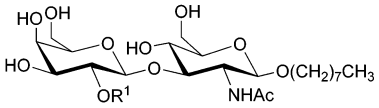


Library A



Library B

The synthesis and screening of two β-D-Gal*p*-(1-3)-β-D-Glc*p*N (lacto-*N*-biose) disaccharide libraries are reported. Solution-phase synthetic modifications at the HO-2′ and NH positions were performed in an effort to enhance the affinity toward galectin-3, a galactose-binding protein involved in tumor metastasis, apoptosis, and inflammation. The libraries were screened for galectin-3 binding by microscale frontal affinity chromatography coupled to mass spectrometry (FAC/MS) allowing for rapid ranking of the different inhibitors and the determination of the galectin-3 binding K_d's. Compounds bearing a hydrophobic substituent on the NH group showed the highest affinity for the lectin. The *N*-naphthoyl derivative (K_d = 10.6 μM) was the best inhibitor with a 7 times increased affinity as compared to the *N*-acetyl parent compound (K_d = 73.3 μM).

Introduction

Galectins[1] are a family of lectins which share certain conserved amino acid sequences in their carbohydrate recognition domain, giving them an affinity for β-galactosides. Widely distributed throughout the animal kingdom (mammals, frogs, fishes, sponges...), these carbohydrate-binding proteins are expressed in a variety of organs and tissues such as lung, liver, and small intestine among others. Galectins are mainly localized in the nucleus and the cell cytoplasm, although they can be secreted from the cell by a nonclassical pathway and interact with appropriately glycosylated proteins at the cell surface or within the extracellular matrix.[2] Here, they are thought to regulate cell−cell and cell−matrix interactions with adhesion molecules such as integrins and matrix glycoproteins such as laminin and fibronectins.[3] A wide variety of biological phenomena have been related to galectins, such as embryonic development, differentiation, tumor metastasis, apoptosis, and RNA splicing.[4] However, relatively little is known about the mechanism by which these lectins exert these functions.

Galectin-3 is one of the most extensively studied members of the galectins. Numerous biological investigations have demonstrated its involvement in tumor metastasis, apoptosis, and inflammatory processes.[5] For example, T cells infected with human leukemia virus type I express a high level of galectin-3, preventing drug-induced apoptosis normally occurring with uninfected cells.[6] Similarly, galectin-3 enhances the metastatic potential of human breast carcinoma cells through antiapoptotic regulation.[7]

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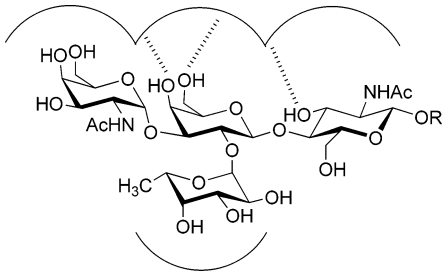

FIGURE 1. Schematic representation of blood group A tetrasaccharide bound to galectin-3 (dashed lines represent key hydrogen bonding).

The X-ray crystal structure of human galectin-3 in complex with lactose and *N*-acetyllactosamine[8] as well as modeling[9] and thermodynamic[10] studies have demonstrated the carbohydrate-binding specificity of this lectin. Although all galectins bind well to terminal galactosides, galectin-3 differs significantly in its recognition of galactosyl residues within oligosaccharides. For example, both galectin-1 and galectin-3 bind the type I and type II disaccharides β-D-Gal*p*-(1-3)-D-Glc*p*NAc and β-D-Gal*p*-(1-4)-D-Glc*p*NAc in the millimolar range. However, galectin-3 appears to have an extended binding site that can accommodate also longer oligosaccharides such as α-Neu*p*5Ac-(2-3)-β-D-Gal*p*-(1-4)-D-Glc*p*NAc or the human blood group A epitope α-D-Gal*p*NAc-(1-3)-[α-L-Fuc*p*-(1-2)]-β-D-Gal*p*-(1-4)-D-Glc*p*NAc that it binds with an almost 100-fold higher affinity compared to galectin-1.[11] A primary carbohydrate binding site could be responsible for the binding of the β-D-Gal*p*-(1-3/4)-D-Glc*p*NAc moiety, and additional subsites could be involved in the recognition of GalNAc and Fuc residues (Figure 1). In the X-ray structure of human galectin-3 in complex with lactose and *N*-acetyllactosamine,[8] the side chain of three amino acids (histidine-168, asparagine-160, and arginine-162, invariant in all galectins sequenced), is hydrogen bonded to Gal HO-4, and a tryptophan (181) is stacked against the sugar ring. The Gal HO-6 is also tightly bound but to neither Gal HO-2 nor Gal HO-3. The GlcNAc moiety is more solvent exposed, and only HO-3 makes direct hydrogen-bond contact with the protein.

Although the structure of galectins is well documented, much work remains to clearly understand the mechanism by which these lectins exert their functions. Specific synthetic inhibitors are valuable tools as probes for protein structure and mechanism; they are also attractive for their potential therapeutic applications. Over the last five years, Gabius et al. have largely explored the affinity of lactose dendrimers for galectins. Strong multivalency effects were first observed with galectin-3 for flexible wedgelike glycoclusters,[12] whereas a positive effect of spacer

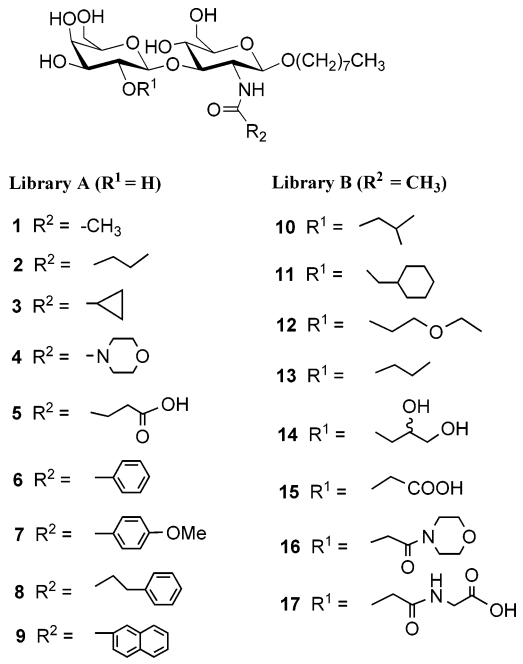

FIGURE 2. Lacto-*N*-biose libraries.

rigidification was further unveiled.[13] On the other hand, Nilsson et al. reported very potent monovalent inhibitors by screening synthetic galactoside libraries. Although 3-(triazol-1-yl)-1-thio-galactosides were found to be efficient small inhibitors,[14] the best ligand of galectin-3 synthesized so far consists of C_2-symmetrical thiodigalactosides bearing benzamido substituents at the C-3 position of the galactosyl unit.[15] The activity of these compounds reached the nanomolar level producing a significant improvement over the previous generation of inhibitors developed by this group around a *N*-acetyllactosamine scaffold.[16]

To further probe the recognition domain, we have focused on the galectin-3 specificity toward type I structures and carried out solution-phase synthesis and screening of two libraries of N- and 2′-functionalized type I disaccharides **1**−**17** (Figure 2). The modifications were performed from commercially available reagents to ensure a large molecular diversity in terms of flexibility, polarity, or steric hindrance of the new functions. Library **A** was prepared upon O-alkylation of a selectively protected disaccharide affording a stable ether linkage, whereas library **B** was obtained through N-acylation. Each molecule prepared in this study was voluntarily different from the other to facilitate the identification of potent inhibitors by their specific molecular weight in a screening approach based on microscale affinity chromatography coupled to electrospray mass spectrometry (FAC/MS).[17] This high-throughput screening technique developed by our group has already proven to be very efficient

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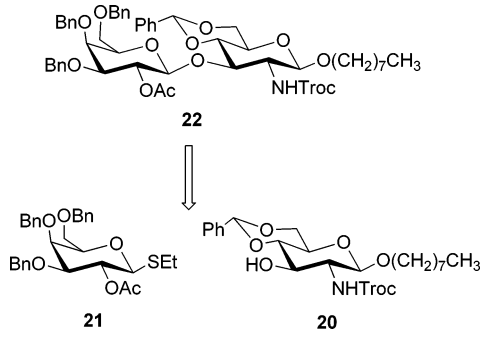
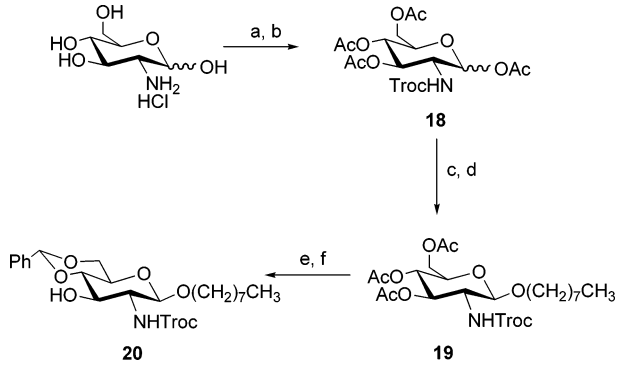
SCHEME 1. Retrosynthesis of Disaccharide 22



SCHEME 2. Synthesis of 20[a]



(a) TrocCl (1.2 equiv), aqueous $NaHCO_3$ (5 equiv); (b) Ac_2O, pyridine (95% over two steps); (c) HBr (33%, AcOH), CH_2Cl_2; (d) octyl alcohol, $Hg(CN)_2$, CH_2Cl_2 (90% over two steps); (e) MeONa/MeOH (5 mM); (f) α,α-dimethoxytoluene, CSA cat., CH_3CN (80% over two steps).

for the evaluation of carbohydrate−protein binding interactions with lectins[18] or antibodies[19] and was recently used to screen a mixture of 1000 modified trisaccharides[20] targeting *N*-acetylglucosaminyltransferase(V) (GlcNAcT-V or Mgat5), an enzyme regulating the branching pattern of N-linked oligosaccharides on glycoproteins. In the context of the present work on the development of inhibitors for galectin-3, it is interesting to note that GlcNAcT-V initiates the oligosaccharide branch that carries the ligands for this galectin at the surface of T-cells.[21]

Results and Discussion

1. Synthesis of Lacto-*N*-biose Libraries. The disaccharide **22** (Scheme 1) was designed as a versatile precursor for the library syntheses. The hydrophobic octyl aglycon would allow for easy purification of the final compounds by reversed solid-phase extraction, and it would also increase the ES-MS detection sensitivity. The protecting groups on each building block should ensure selective β-glycosylation by the neighboring participating group and allow for selective deprotection of HO-2 and/or NH functions for further derivatization. The synthesis of **22** was performed by glycosylation of the acceptor **20** with the known thioglycoside donor **21**.[22]

For the synthesis of **20** (Scheme 2), commercially available D-glucosamine hydrochloride was first converted into **18** by reaction with (2,2,2-trichloroethoxy)carbonyl chloride in aqueous hydrogenocarbonate followed by acetylation according to a literature procedure.[23] Treatment of the mixture of anomeric acetates with hydrogen bromide gave a glycosyl bromide which was used directly in a mercuric cyanide-promoted[24] glycosylation of octyl alcohol to give **19** in 90% yield. Next, conditions for removal of the *O*-acetyl groups were investigated. It was found that mild Zemplén conditions (5 mM MeONa in methanol, 0 °C) converted **19** into the expected free *N*-Troc monosaccharide, without any concomitant transformation of the *N*-Troc group. The crude product was reacted with α,α-dimethoxytoluene and camphorsulfonic acid (CSA) in acetonitrile to give **20** (80% yield).

Among three different donors tested for the next glycosylation step, the thioethyl galactoside **21**[22,25] gave the best result. Reaction between **20** and **21** promoted by NIS/AgOTf[26] in CH_2Cl_2 occurred at low temperature (−70 °C/−50 °C) affording the expected disaccharide **22** in 67% yield (Scheme 3). The β-thiophenyl analog[27] of **21** gave a mixture of α and β anomers (46%, 3:1 β/α) when reacted with **20** under similar conditions, whereas the corresponding α-galactosyl bromide gave yields not exceeding 30% when reacted with **20** under Lemieux conditions.[28] The stereochemistry of the newly formed glycosidic bond was confirmed by ^{1}H NMR spectroscopy ($J_{1,2}$ = 8.4 Hz for H-1′ in **22**), and the 1−3 linkage type was confirmed[29] by the upfield shift (83.3 ppm) of the C-3 signal in the final product **1**. The unexpected formation of α-disaccharide from the β-thiophenyl donor might be explained by in situ anomerization of the donor prior to glycosylation. Indeed, thioglycosides have been demonstrated to equilibrate between α and β anomers in the presence of catalytic iodonium ions.[30] This side reaction was probably only detected when using the thiophenyl donor because of its lowest reactivity compared to **21**.

Selective de-O-acetylation of **22** (NaOMe) was problematic because the prolonged reaction time necessary for completion of the reaction led also to transesterification of the *N*-Troc group with methanol. Therefore, the *N*-Troc group of **22** was first selectively removed with freshly activated Zn in AcOH[25] affording the free amino compound **23** (75%). It is noteworthy that neither acetal hydrolysis nor O−N acetyl migration was observed during these reaction conditions. Derivative **23** was used for generation of library **A** by N-acylation with a range of commercially available acetyl chlorides and carboxylic anhydrides in pyridine. After removal of the benzylidene and benzyl groups by hydrogenolysis over 20% $Pd(OH)_2$/C followed by

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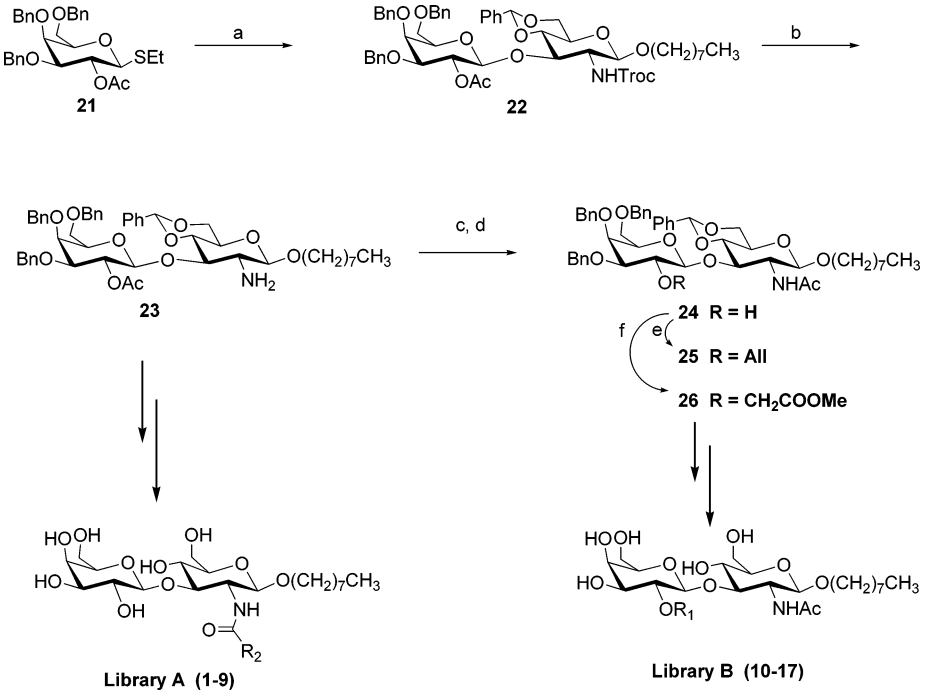
SCHEME 3. Synthesis of HO-2′ and NH Derivatized Lacto-*N*-biose Derivatives[a]



(a) **20** (1 equiv), NIS (1 equiv), AgOTf (0.2 equiv), CH_2Cl_2 (67%); (b) Zn, AcOH (75%); (c) Ac_2O, pyridine; (d) MeONa/MeOH (85% over two steps); (e) AllBr (1.5 equiv), NaH (2 equiv), DMF (87%); (f) $BrCH_2COOMe$ (1.5 equiv), NaH (2 equiv), DMF (35%).

Zemplén de-O-acetylation and purification by solid-phase extraction with C-18 Sep-Pak cartridges, compounds **1**−**9** (30−90%) were isolated and characterized by MS and ^{1}H NMR (Table 1). For the de-O-acetylation of the succinic derivative **5**, Zemplén conditions were replaced by hydrolysis with aqueous sodium hydroxide to avoid cyclization of the amine into the corresponding succinimide.

Library **B** was prepared starting from the de-O-acetylated (MeOH/MeONa) *N*-acetyl derivative **24**. Direct alkylation of **24** at HO-2′ was performed in DMF using NaH and commercially available alkyl bromides. Because of the steric hindrance of this position, the yields usually did not exceed 35% except with allyl bromide (87%); however, intact starting materials could be recovered and reengaged in the next reaction. Attempts to improve the yield by using excess amounts of NaH and bromides led to untractable mixtures, probably because of N-alkylation side reactions and partial decomposition of the products. Compounds **10**−**12** (16−35%) were obtained after alkylation with the corresponding bromides, hydrogenolysis, and purification as described for library **A**. The allyl derivative **25** was oxidized by methylmorpholine *N*-oxide and OsO_4[31] to give the racemic diol **14** (75% from **25**) after deprotection, whereas the propyl derivative **13** (100% from **25**) was directly obtained from **25** by hydrogenolysis. The methyl ester **26** was a versatile intermediate for further elongation of the disaccharide. Saponification of **26** followed by conversion into the *p*-nitrophenyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC)[32] afforded derivatives **16** and **17** (61 and 63%, respectively) upon condensation with morpholine and glycine methylester hydrochloride.

2. Screening of the Lacto-*N*-biose Libraries. Our group recently introduced the use of frontal affinity chromatography coupled to electrospray mass spectrometry (FAC/MS) as a rapid and efficient technique for the screening of glycoconjugate libraries.[17,20] Requiring a very small amount of receptor and analyte, this method allows the ranking of protein ligands or inhibitors from a library in a single analysis and also gives access to their dissociation constants (K_d). A microscale tubing column is prepared by immobilizing a biotinylated galectin-3 onto streptavidin controlled-pore glass (CPG) beads. A solution containing the library and a void volume marker in ammonium acetate buffer is continuously infused through the column, and the effluent is entered into an electrospray mass spectrometer to detect each component by selected-ion monitoring. The order of elution of compounds from the column parallells their affinities for the receptor, with the tightest-binding ligands eluting last. The dissociation constants of individual active ligands in these mixtures can be determined from eq 1 according to the frontal affinity chromatography theory first developed by Kasai et al.[33]

$$V_x - V_0 = B_t/(K_d + [X]_0) \quad (1)$$

B_t, the column capacity, is the amount of immobilized protein that can bind the ligand, and K_d is the dissociation constant. $[X]_0$ is the ligand concentration, V_x is the elution volume of ligand X, and V_0 is the void volume of the system. The B_t and K_d values are calculated from a nonlinear regression analysis of retention volumes as a function of ligand concentration.

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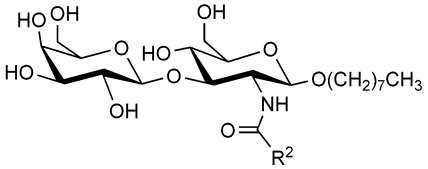
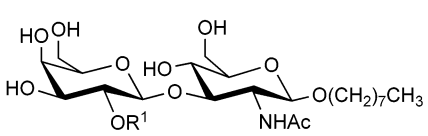
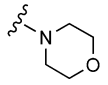
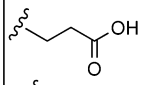
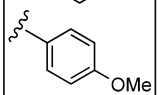
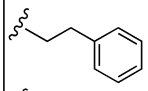
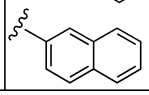
TABLE 1. HRMS and ^{1}H NMR of Libraries A and B

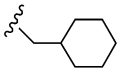
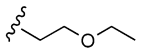
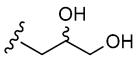
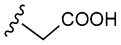
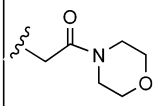
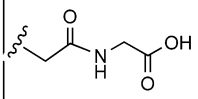
Library A



Library B



Cpnd	R^2	M calcd	$[M+Na]^+$ found	δ ppm
1	CH_3	495.27	518.26	4.55*, 4.43* [a]
2		523.30	546.28	4.99*, 4.84*, 2.70, 2.10, 1.44 [b]
3		521.28	544.27	4.48*, 4.28*, 0.88, 0.73 [c]
4		566.31	589.29	4.51*, 4.25* [c]
5		553.27	576.26	5.01*, 4.87*, 3.11-2.97 [b]
6		543.27	580.27	8.32-7.98, 5.15*, 4.85* [b]
7		573.28	610.28	8.72, 7.94, 5.57*, 5.24* [b]
8		585.68	608.30	7.79, 4.98*, 4.83*, 3.40, 3.01 [b]
9		607.30	630.29	8.90-7.64, 5.21*, 4.90* [b]

Cpnd	R^1	M calcd	$[M+Na]^+$ found	δ ppm
10		551.53	574.32	4.39*, 4.33*, 1.83, 0.89 [c]
11		591.36	614.35	4.36*, 4.32* [c]
12		567.33	590.31	4.55*, 4.34*, 1.21 [c]
13		537.31	560.30	4.37*, 4.34*, 1.55 [c]
14		569.30	592.29	4.95 (2H)* [b]
15		553.27	576.23	4.42 (2H)*, 4.30, 4.15 [c]
16		622.33	645.32	4.40*, 4.33* [c]
17		610.29	633.28	4.45*, 4.43*, 4.34, 4.17 [c]

*H-1 and H-1′ (J = 7−8.1 Hz). [a] D_2O. [b] D_2O/CD_3CN (1:1 v/v). [c] CD_3OD.

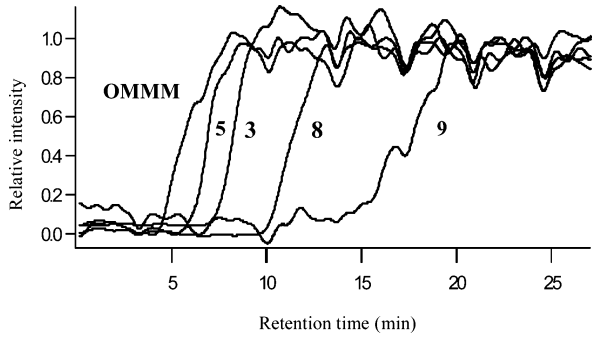


FIGURE 3. Extracted ion chromatograms of a mixture of disaccharides **3**, **5**, **8**, and **9**.

The synthesized libraries were screened by FAC/MS on a recombinant human galectin-3[34] column. The trisaccharide octyl α-D-Man*p*-(1-3)-[α-D-Man*p*-(1-6)]-α-D-mannopyranoside (OMMM), which does not have an affinity for galectin-3, was used as the void volume marker, and the disaccharide **1** was chosen as a standard ligand. Basically, libraries **A** and **B** were split into four mixtures containing an equimolar ratio of each component. The samples were infused into a 10-cm column to determine the strongest inhibitors (Figure 3).

To ensure that the result was not affected by nonspecific binding, a control experiment was performed with a blank biotin-streptavidin CPG bead column of the same length. After correction from subtraction of the blank column experiment, all the compounds synthesized showed an increased affinity compared to the parent disaccharide **1**. Derivatives **9** and **11** appeared, respectively, to be the best inhibitors out of libraries **A** and **B** (Table 2).

TABLE 2. Relative Retention Time of Library Components (2.5−3.5 μM)

library A	relative retention time	library B	relative retention time
1	1.0	**10**	1.6
2	2.0	**11**	3.8
3	2.5	**12**	2.7
4	1.2	**13**	1.7
5	1.2	**14**	2.6
6	2.6	**15**	1.6
7	2.6	**16**	2.6
8	4.9	**17**	1.9
9	11.0		

To make sure that **9** was a competitive inhibitor, the parent disaccharide **1** was infused at the same concentration (3 μM) on the galectin-3 column which had been previously saturated with a solution of **9** (3 μM). As expected, the retention time of **1** observed on the inhibitor-saturated column was decreased 2-fold, clearly indicating a lower population of the free binding site due to the presence of the inhibitor. The K_d values of **1** (73.3 μM) and **9** (10.6 μM) calculated from eq 1 finally indicated

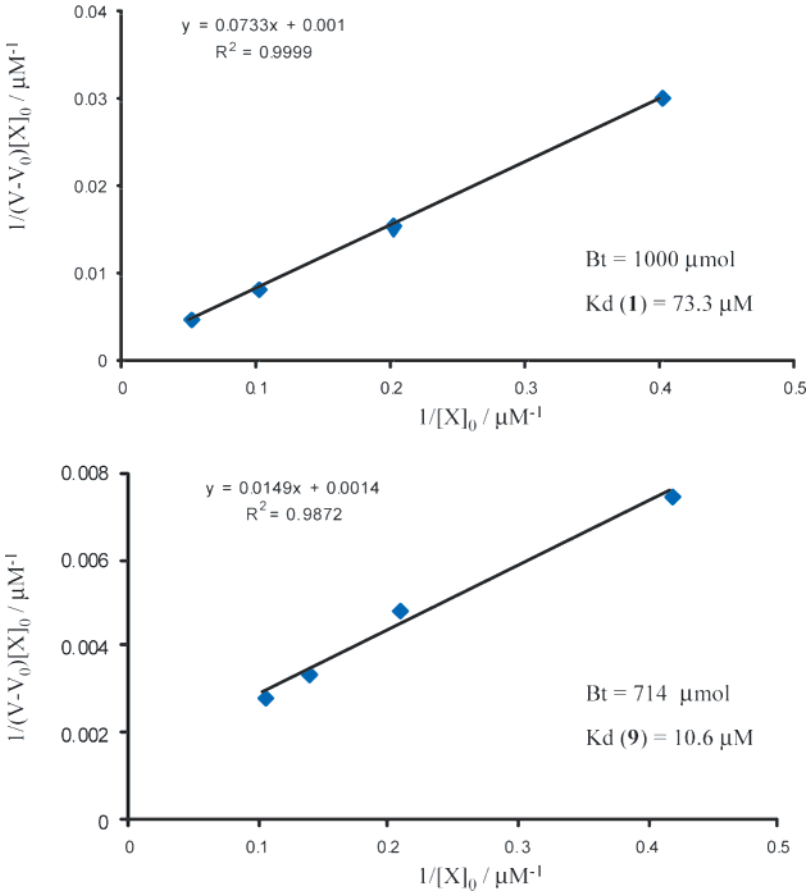


FIGURE 4. Determination of K_d constants for **1** and **9**.

that **9** has a 7-fold increased affinity for galectin-3 compared to **1** (Figure 4).

The screening of library **A** demonstrates that disaccharides bearing N-linked aromatic substituents in the 2-position show enhanced affinity for galectin-3. The strong binding of **9** could result from a stabilizing π-cation interaction[35] between the naphthoyl and the guanidino headgroup of Arg-186 that makes van der Waals contacts with the methyl of the *N*-acetyl group in the LacNAc complex.[8] Indeed, arginine−arene interactions involving Arg-186 have been previously demonstrated by Nilsson et al.[15] to provide substantial affinity enhancement in the design of monovalent galectin-3 inhibitors. A structure/affinity relationship for the compounds derivatized on HO-2′ is less evident. The cyclohexane group of **11** could mimic the fucosyl unit of the blood group A tetrasaccharide and may interact with the lectin by stacking with a close aromatic residue. Trp-181 which sits below the galactosyl unit in the LacNAc complex[8] could be involved in such stabilization. However, in the absence of crystallographic data about the topology of the extended ligand binding site that accommodates the fucosyl unit, further modeling of the complex of **11** and galectin-3 appears necessary to provide information on the surface complementarity between the cyclohexyl ring and the lectin. On the other hand, more polar substituents on **10**, **14**, and **16** also, to a lesser extent, improved the binding to galectin-3 compared to the parent disaccharide **1**, suggesting possible hydrogen bonding to amino acids. However, none of the substitutions in this position seemed to affect key amino acid binding because no compound exhibited a large binding improvement. Undoubtedly, further structural studies of the complexes by modeling and X-ray crystallography are now necessary for rationalization of the enhanced inhibitory of compounds **9** and **11** and for the design of more potent inhibitors.

In summary, we have demonstrated, by screening libraries of N- and 2′-derivatized type I disaccharides with the FAC/MS technique, that modifications at either the N- or 2′-position resulted in an enhanced affinity for galectin-3. The best result was obtained with the *N*-naphthoyl derivative **9** ($K_d = 10.6\ \mu$M), which showed a 7-fold increased affinity compared to the *N*-acetyl parent compound **1** ($K_d = 73.3\ \mu$M). A less significant affinity enhancement could be seen with the compounds modified at the 2′-position. However, new compounds modified at both the 2- and 2′-positions should be interesting future candidates for high-affinity galectin-3 ligands.

Experimental Section

General. Column chromatography was performed on Silica Gel 60 (40−63 μM). TLC was performed on Silica Gel 60-F254 with detection by charring with H_2SO_4/MeOH/H_2O (3:45:45 v/v/v). ^{1}H NMR spectra were recorded at 300, 400, or 600 MHz, and ^{13}C NMR spectra were recorded at 100 MHz. Assignments of proton resonances were aided by 2-D COSY experiments. Pyridine, dichloromethane, and methanol were distilled over the appropriate drying agent (KOH, CaH, and Mg/I_2, respectively). Acetonitrile, DMF, and octanol were dried over 4 Å molecular sieves before use. Compounds **18**[23] and **21**[22,25] were prepared as described. The purity of compounds **1**−**17** was judged by ^{1}H NMR to be ≥95%.

(35) Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303−1324.

Octyl 3,4,6-tri-*O*-Acetyl-2-deoxy-2-[[(2,2,2-trichloroethoxy)-carbonyl]amino]-β-D-glucopyranoside (19). A solution of HBr in AcOH (33%, 15 mL) was added to a solution of **18** (3 g, 5.74 mmol) in CH_2Cl_2 (30 mL) at 0 °C. The mixture was stirred at 0 °C for 30 min and then at room temperature for 1.5 h and was thereafter poured into stirred ice−water. The organic phase was separated, and the aqueous phase was extracted with CH_2Cl_2. The combined organic phases were washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated. The residue was taken up in CH_2Cl_2 (40 mL) containing dried octanol (1.08 mL, 6.89 mmol) and $Hg(CN)_2$ (1.6 g, 6.31 mmol), and the mixture was stirred overnight at room temperature, then filtered through Celite, washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated. Recrystallization of the residue from hexane gave **19** as a white solid (3.08 g, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.28 (t, 1H, *J* = 10 Hz, H-3), 5.03 (t, 1H, *J* = 10 Hz, H-4), 4.74 and 4.64 (2d, 2H, *J* = 11 Hz, Cl_3CCH_2), 4.61 (d, 1H, *J* = 7.8 Hz, H-1), 4.25 (dd, 1H, *J* = 4.8, 12.3 Hz, H-6a), 4.10 (dd, 1H, *J* = 2.5, 12.3 Hz, H-6b), 3.85 (dt, 1H, *J* = 6.6, 9.6 Hz, Oct-OCHH), 3.65, (m, 1H, H-5), 3.56 (dd, 1H, *J* = 7.8, 10 Hz, H-2), 3.44 (dt, 1H, *J* = 6.6, 9.6 Hz, Oct-OCHH), 2.05 (2s, 9H, $OCOCH_3$), 1.54 (m, 2H, Oct-OCH_2CH_2), 1.23 (m, 10H, Oct-CH_2), 0.85 (t, 3H, *J* = 7 Hz, Oct-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 170.4, 169.3, 153.8, 100.7, 95.4, 74.5, 71.9, 71.7, 70.4, 68.8, 62.2, 56.4, 31.9, 29.5, 29.4, 29.3, 25.9, 22.7, 20.8, 20.72, 20.70, 14.2. Anal. Calcd for $C_{23}H_{36}Cl_3NO_{10}$: C, 46.59; H, 6.12; Cl, 17.94; N, 2.36. Found: C, 46.42; H, 5.98; Cl, 17.73; N, 2.30.

Octyl 4,6-*O*-Benzylidene-2-deoxy-2-[[(2,2,2-trichloroethoxy)-carbonyl]amino]-β-D-glucopyranoside (20). Compound **19** (5.095 g, 8.5 mmol) in MeOH (50 mL) was de-O-acetylated at 0 °C by addition of a solution of 1 M NaOMe in MeOH (250 μL). When TLC indicated completion of the reaction, the solution was neutralized by addition of Amberlite IR 120 (H^+) resin, then filtered and concentrated. The residue was dissolved in CH_3CN (40 mL), whereafter α,α-dimethoxytoluene (2.5 mL, 17 mmol) and camphorsulfonic acid (380 mg, 1.7 mmol) were added. The solution was stirred overnight at room temperature, then neutralized with Et_3N, and concentrated. Purification of the residue by flash chromatography (toluene/ethyl acetate 8:2 v/v) gave **20** (3.75 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50−7.35 (m, 5H, H arom), 5.51 (s, 1H, CHPh), 5.28 (d, 1H, *J* = 8 Hz, NH), 4.71 (m, 2H, Cl_3CCH_2), 4.56 (bd, 1H, *J* = 8 Hz, H-1), 4.31 (dd, 1H, *J* = 5, 10.5 Hz, H-6a), 4.04 (m, 1H, H-3), 3.83 (dt, 1H, *J* = 6.8, 9.6 Hz, Oct-OCHH), 3.75 (t, 1H, *J* = 10.5 Hz, H-6b), 3.52 (t, 1H, *J* = 9.2 Hz, H-4), 3.41 (m, 3H, Oct-OCHH, H-2, 5), 1.54 (m, 2H, Oct-OCH_2CH_2), 1.28 (m, 10H, Oct-CH_2), 0.88 (t, 3H, *J* = 7 Hz, Oct-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.5, 137.0, 129.3, 128.4, 128.3, 126.3, 101.8, 100.9, 95.4, 81.4, 74.6, 70.6, 70.4, 68.6, 66.0, 58.8, 31.8, 29.5, 29.3, 29.2, 25.8, 22.6, 14.0. Anal. Calcd for $C_{24}H_{34}Cl_3NO_7$: C, 51.95; H, 6.18; Cl, 19.17; N, 2.52. Found: C, 51.85; H, 6.26; Cl, 18.70; N, 2.53.

Octyl 3-*O*-(2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-4,6-*O*-benzylidene-2-deoxy-[[(2,2,2-trichloroethoxy)carbonyl]amino]-β-D-glucopyranoside (22). To a stirred and cooled (−70 °C) solution of **20** (997 mg, 1.8 mmol) and **21** (970 mg, 1.8 mmol) in CH_2Cl_2 (20 mL) were successively added NIS (405 mg, 1.8 mmol) and AgOTf (91 mg, 0.36 mmol). The mixture was stirred at −50 to −70 °C until TLC indicated total disappearance of **21** (3 h), then neutralized with Et_3N, and filtered through Celite. The solution was washed with saturated aqueous $NaHCO_3$ and aqueous $Na_2S_2O_3$, dried over Na_2SO_4, and concentrated. Purification by flash chromatography (petroleum ether/ethyl acetate 7:3 v/v) afforded **22** (1.245 g, 67%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.45−7.16 (m, 20H, H arom), 5.47 (s, 1H, CHPh), 5.29 (dd, 2H, *J* = 7.8, 10.2 Hz, H-2′, NH), 4.80 (m, 3H, H-1, Cl_3CCH_2, CH_2Ph), 4.58, 4.54, 4.40 (3d, 4H, *J* = 12 Hz, Cl_3CCH_2, CH_2Ph), 4.51 (d, 1H, *J* = 8.4 Hz, H-1′), 4.31 (m, 1H, H-3), 4.28 (dd, 1H, *J* = 4.8, 10.5 Hz, H-6a), 4.20 (m, 2H, CH_2Ph), 3.87 (d, 1H, *J* = 2.5 Hz, H-4′), 3.80 (dt, 1H, *J* = 6.6, 9.6 Hz, Oct-OCHH), 3.73 (t, 1H, *J* = 10.5 Hz, H-6b), 3.64 (t, 1H, *J* = 9.5 Hz, H-4), 3.55 (t, 1H, *J* = 8.5 Hz, H-6′a), 3.41 (m, 2H, Oct-OCHH, H-5), 3.38 (dd, 1H, *J* = 2.5, 10.5 Hz, H-3), 3.33 (m, 1H, H-5′), 3.28 (m, 1H, H-6′b), 3.15 (m, 1H, H-2), 1.95 (s, 3H, $OCOCH_3$), 1.53 (m, 2H, Oct-OCH_2CH_2), 1.27 (m, 10H, Oct-CH_2), 0.86 (t, 3H, *J* = 7 Hz, Oct-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.4, 153.6, 138.5−126.0, 101.1, 100.0, 95.5, 80.3, 80.2, 77.2, 74.4, 74.3, 73.4, 73.0, 72.2, 71.8, 71.7, 70.5, 68.7, 68.2, 66.1, 58.4, 31.8, 29.5, 29.3, 29.2, 25.8, 22.6, 20.9, 14.0. Anal. Calcd for $C_{53}H_{64}Cl_3NO_{13}$: C, 61.84; H, 6.27; Cl, 10.33; N, 1.36. Found: C, 61.58; H, 6.30; Cl, 10.36; N, 1.35.

Octyl 3-*O*-(2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-2-amino-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (23). Compound **22** (1.245 g, 1.2 mmol) was dissolved in AcOH, and freshly activated zinc dust (1 g) was added to the stirred solution. After 4 h stirring at room temperature, the reaction mixture was filtered through Celite, concentrated, and coevaporated with toluene (3 times). Purification by flash chromatography (petroleum ether/ethyl acetate/triethylamine 7:3:0.1%) afforded **23** (768 mg, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42−7.15 (m, 20H, H arom), 5.46 (s, 1H, CHPh), 5.32 (dd, 1H, *J* = 8, 10 Hz, H-2′), 4.87 (d, 1H, *J* = 11.7 Hz, CH_2Ph), 4.59 (d, 1H, *J* = 12.2 Hz, CH_2Ph), 4.58 (d, 1H, *J* = 8 Hz, H-1′), 4.55 (d, 1H, *J* = 11.7 Hz, CH_2Ph), 4.41 (d, 1H, *J* = 12.3 Hz, CH_2Ph), 4.25 (d overlapped, 2H, *J* = 8.1 Hz, H-1, 6a), 4.18 (s, 2H, CH_2Ph), 3.89 (d, 1H, *J* = 3 Hz, H-4′), 3.85 (dt, 1H, *J* = 6.6, 9.3 Hz, Oct-OCHH), 3.76 (t, 1H, *J* = 10.5 Hz, H-6b), 3.72 (t, 1H, *J* = 9 Hz, H-4), 3.61 (t, 1H, *J* = 9 Hz, H-3), 3.59 (t, 1H, *J* = 7.8 Hz, H6′a), 3.48 (dt, 1H, *J* = 6.6, 9.3 Hz, Oct-OCHH), 3.42 (dd, 1H, *J* = 3, 10 Hz, H-3′), 3.37−3.22 (m, 3H, H-5, 5′, 6′b), 2.92 (dd, 1H, *J* = 8.1, 9 Hz, H-2), 2.00 (s, 3H, $OCOCH_3$), 1.59 (m, 2H, Oct-OCH_2CH_2), 1.29 (m, 10H, Oct-CH_2), 0.87 (t, 3H, *J* = 7 Hz, Oct-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 138.4, 137.9, 137.7, 137.5, 129.0−126.0, 104.0, 101.5, 101.3, 82.1, 80.6, 80.2, 74.5, 73.4, 73.1, 72.4, 72.1, 71.7, 70.3, 68.8, 68.1, 66.6, 56.6, 31.8, 29.6, 29.3, 29.2, 25.9, 22.6, 21.2, 14.0. Anal. Calcd for $C_{50}H_{63}NO_{11}$: C, 70.32; H, 7.44; N, 1.64. Found: C, 69.93; H, 7.54; N, 1.63.

Octyl 2-Acetamido-3-*O*-(3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (24). A solution of **23** (450 mg, 0.53 mmol) in pyridine/acetic anhydride (4:1 v/v, 5 mL) was stirred at room temperature for 30 min, concentrated, and coevaporated with toluene. The residue was dissolved in MeOH/CH_2Cl_2 (3:1 v/v, 40 mL) and de-O-acetylated at room temperature by addition of 1 M MeONa in MeOH (1 mL). The reaction mixture was neutralized with Amberlite IR 120 (H^+) resin, filtered, and concentrated. Purification by flash chromatography (petroleum ether/ethyl acetate 6:4 v/v) afforded **24** (380 mg, 85%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.45−7.21 (m, 20H, H arom), 5.95 (d, 1H, *J* = 7.2 Hz, NH), 5.50 (s, 1H, CHPh), 4.87 (d, 1H, *J* = 8.4 Hz, H-1), 4.86 (d, 1H, *J* = 11.4 Hz, CH_2Ph), 4.59 (m, 3H, CH_2Ph), 4.36−4.27 (d overlapped, 5H, *J* = 7.8 Hz, H-1′, 3, 6a, CH_2Ph), 3.94 (dd, 1H, *J* = 7.8, 9.6 Hz, H-2′), 3.87 (d, 1H, *J* = 3 Hz, H-4′), 3.81 (dt, *J* = 6.6, 9.6 Hz, Oct-OCHH), 3.74 (t, 1H, *J* = 10 Hz, H-6b), 3.62 (t, 1H, *J* = 9.6 Hz, H-4), 3.56 (t, 1H, *J* = 7.8 Hz, H-6′a), 3.51 (bt, 1H, *J* = 5.4 Hz, H-5′), 3.47−3.37 (m, 4H, H-2, 5, 6′b, Oct-OCHH), 3.31 (dd, 1H, *J* = 3, 9.6 Hz, H-3′), 1.86 (s, 3H, $NCOCH_3$), 1.54 (m, 2H, Oct-OCH_2CH_2), 1.25 (m, 10H, Oct-CH_2), 0.85 (t, 3H, *J* = 7 Hz, Oct-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 138.7, 138.4, 137.7, 137.0, 128.9−126.1, 103.3, 101.3, 101.1, 81.7, 79.9, 76.2, 74.6, 73.5, 73.4, 73.2, 72.4, 70.6, 70.2, 68.7, 68.3, 66.2, 57.1, 31.8, 29.5, 29.3, 29.2, 25.8, 23.5, 22.6, 14.0. Anal. Calcd for $C_{50}H_{63}NO_{11}$: C, 70.32; H, 7.44; N, 1.64. Found: C, 69.97; H, 7.36; N, 1.70.

General Procedure for the Preparation of Library A. A solution of **23** (0.035 mmol) and acyl chloride or carboxylic anhydride (0.175 mmol, 5 equiv) in pyridine (3 mL) was stirred overnight at room temperature. The excess reagent was destroyed by addition of MeOH (1 mL) at 0 °C, and the solution was then concentrated. After purification by flash chromatography (toluene/ethyl acetate), the protected disaccharides were dissolved in MeOH/

THF (1:1 v/v). The solution was stirred overnight under a stream of hydrogen in the presence of 20% $Pd(OH)_2/C$ (50 mg), filtered through Celite, and concentrated. The residue, dissolved in MeOH (1 mL), was de-O-acetylated at room temperature by addition of 1 M NaOMe solution in MeOH (10 μL). The mixture was neutralized with Amberlite IR 120 (H^+) resin, filtered, and concentrated. The residue was dissolved in the minimum volume of CH_3CN/H_2O (1:1 v/v), loaded onto a C_{18} Sep-Pak cartridge (previously equilibrated with H_2O), washed with water, and finally eluted with CH_3CN/H_2O (1:1 v/v) to give **1** (85%), **2** (74%), **3** (79%), **4** (30%), **5** (75%), **6** (90%), **7** (80%), **8** (60%), and **9** (80%).

For **1**: ^{13}C NMR (100 MHz, D_2O) δ 175.2, 104.3, 101.7, 83.3, 76.2, 76.1, 73.3, 71.5, 71.4, 69.6, 69.4, 61.9, 61.6, 55.5, 32.0, 29.5, 29.4, 29.2, 26.0, 23.2, 22.9, 14.3.

For **9**: ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.8, 134.0, 132.3, 132.0, 128.6−124.2, 103.5, 100.6, 84.2, 76.5, 75.7, 72.7, 70.6, 68.7, 68.5, 68.1, 60.8, 60.5, 54.8, 31.0, 29.0, 28.8, 28.6, 25.5, 21.8, 13.7.

General Procedure for the Preparation of Library B. Sodium hydride dispersion (80% in oil, 0.12 mmol, 2 equiv), was added at 0 °C to a stirred solution of **24** (0.06 mmol) and alkyl bromide (0.09 mmol, 1.5 equiv) in DMF (2 mL). The mixture was allowed to attain room temperature, and after 12 h, MeOH was added to destroy the excess of reagents. The solution was concentrated, and the residue was dissolved in ethyl acetate. The solution was washed with brine, dried over Na_2SO_4, and concentrated. Flash chromatography (toluene/ethyl acetate) followed by hydrogenolysis over 20% $Pd(OH)_2/C$ and final purification by solid-phase extraction with a C_{18} Sep-Pak cartridge as described before afforded **10** (16%), **11** (28%), and **12** (35%).

Octyl 2-Acetamido-3-*O*-(2-*O*-allyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (25). This compound was obtained (87%) following the general procedure for O-alkylation described before. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.44−7.19 (m, 20H, H arom), 5.95 (m, 2H, OCH_2CH=CH_2, NH), 5.48, (s, 1H, C*H*Ph), 5.27 (dd, 1H, J = 1.8, 17.4 Hz, OCH_2-CH=CH_2), 5.24 (d, 1H, J = 8.4 Hz, H-1), 5.14 (dd, 1H, J = 1.8, 11 Hz, OCH_2CH=CH_2), 4.85 (d, 1H, J = 11.4 Hz, CH_2Ph), 4.63 (s, 2H, CH_2Ph), 4.56 (d, 1H, J = 11.4 Hz, CH_2Ph), 4.54 (t, 1H, J = 9.6 Hz, H-3), 4.33 (d, 1H, J = 7.8 Hz, H-1′), 4.28 (m, 5H, H-6a, CH_2Ph, OCH_2CH=CH_2), 3.85 (d, 1H, J = 3 Hz, H-4′), 3.81 (dt, 1H, J = 6.6, 9.6 Hz, Oct-OC*H*H), 3.72 (t, 1H, J = 10 Hz, H-6b), 3.68 (dd, 1H, J = 7.8, 9.6 Hz, H-2′), 3.59 (t, 1H, J = 9.6 Hz, H-4), 3.55 (t, 1H, J = 10 Hz, H-6′a), 3.48 (m, 2H, H-5, Oct-OCH*H*), 3.38 (m, 2H, H-5′, 6′b), 3.35 (dd, 1H, J = 3, 10 Hz, H-3′), 3.00 (dd, 1H, J = 8.4, 10 Hz, H-2), 1.89 (s, 3H, $NCOCH_3$), 1.55 (m, 2H, Oct-OCH_2CH_2), 1.25 (m, 10H, Oct-CH_2), 0.87 (t, 3H, J = 7 Hz, Oct-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 138.7, 138.4, 137.9, 137.5, 135.7, 128.7−126.2, 116.4, 103.5, 100.9, 99.7, 82.3, 79.9, 79.0, 77.6, 74.5, 73.8, 73.4, 73.2, 72.8, 72.5, 70.3, 68.7, 68.3, 66.1, 59.5, 31.8, 29.5, 29.3, 29.2, 25.8, 23.6, 22.6, 14.0. HR−FABMS calcd for $C_{53}H_{67}NO_{11}$ $[M+Na]^+$, 916.4612; found, 916.4610.

Preparation of 13: Hydrogenation of **25** over 20% $Pd(OH)_2/C$ in MeOH/THF (1:1 v/v) followed by filtration through a C_{18} Sep-Pak cartridge as described before afforded **13** (100% from **25**).

Preparation of 14: Methylmorpholine *N*-oxide (17 mg) and OsO4 (2.5 wt %, 20 μL) were added to a solution of **25** (50 mg, 0.056 mmol) in acetone (1 mL). The mixture was stirred at room temperature overnight, diluted with CH_2Cl_2, washed with water, dried over Na_2SO_4, and concentrated. Purification by flash chromatography (toluene/ethyl acetate 4:6 v/v) followed by hydrogenation and final purification on a C_{18} Sep-Pak cartridge as described before afforded **14** (24 mg, 75%).

Octyl 2-Acetamido-3-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-methoxycarbonylmethyl-β-D-galactopyranosyl)-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (26). This compound was obtained (33%) following the general procedure for O-alkylation described before. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.48−7.18 (m, 20H, H arom), 5.48 (s, 1H, C*H*Ph), 5.35 (d, 1H, J = 7.8 Hz, H-1), 4.82 (d, 1H, J = 11.4 Hz, CH_2Ph), 4.65 (d, 1H, J = 11.4 Hz, CH_2Ph), 4.56 (d, 1H, J = 12 Hz, CH_2Ph), 4.53 (m, 3H, H-3, CH_2Ph, OCH_2COOCH_3), 4.36 and 4.32 (2d, 2H, J = 12 Hz, CH_2Ph), 4.29 (m, 2H, H-1′, 6a), 4.25 (d, 1H, J = 16.2 Hz, OCH_2COOCH_3), 3.93 (d, 1H, J = 3 Hz, H-4′), 3.80 (dt, 1H, J = 6.6, 9.6 Hz, Oct-OC*H*H), 3.70 (m, 4H, H-6b, OCH_2COOCH_3), 3.62 (m, 2H, H-2′, 6′a), 3.54−3.47 (m, 4H, H-4, 5, 5′, Oct-OCH*H*), 3.43 (dd, 1H, J = 5.4, 8.4 Hz, H-6′b), 3.40 (dd, 1H, J = 3, 9.6 Hz, H-3′), 2.92 (ddd, 1H, J = 7.8, 8.4, 10.2 Hz, H-2), 1.93 (s, 3H, $NCOCH_3$), 1.55 (m, 2H, Oct-OCH_2CH_2), 1.28 (m, 10H, Oct-CH_2), 0.87 (t, 3H, J = 7 Hz, Oct-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 171.5, 138.6, 137.9, 137.8, 137.6, 128.5−126.1, 103.4, 100.5, 99.7, 82.6, 80.6, 79.6, 78.1, 74.6, 73.4, 72.8, 72.7, 71.8, 70.7, 70.4, 68.7, 67.9, 66.1, 59.8, 51.9, 31.8, 29.6, 29.3, 29.2, 25.8, 23.3, 22.6, 14.0. HR−FABMS calcd for $C_{53}H_{67}NO_{13}$ $[M+Na]^+$, 948,4510; found, 948.4515.

Preparation of 15: 26 was dissolved in aqueous NaOH (0.25 M, 1 mL) and stirred for 1 h at room temperature. The solution was neutralized with Amberlite IR 120 (H^+) and concentrated. The residue was purified using a C_{18} Sep-Pak cartridge as described before to give **15** (91%).

Preparation of 16 and 17: Saponification of **26** was realized as described for **15**. The crude carboxylic acid intermediate was coevaporated with toluene and dried under vacuum for 5 h. The residue was dissolved in DMF, and *p*-nitrophenol (5 equiv) and EDAC (5 equiv) were added. The mixture was stirred at room temperature for 5 h, and the amine (2 equiv) was added. The solution was concentrated, and the residue was dissolved in ethyl acetate. The solution was washed with brine, dried over Na_2SO_4, and concentrated. Flash chromatography (toluene/ethyl acetate), hydrogenolysis, and C_{18} Sep-Pak purification afforded **16** (29 mg, 61%), whereas supplementary hydrolysis of the glycine methyl ester with 0.25 M aqueous NaOH before final purification gave **17** (18 mg, 63%).

FAC/MS Assay. Preparation of the Column. A biotinylation reagent, prepared just before use by dissolving sulfo-NHS-LC-Biotin (7.2 μM, 10 μL) in sodium bicarbonate buffer (pH 8.62, 40 mM with 20 mM lactose) was added to a solution of freeze-dried galectin-3 in sodium bicarbonate buffer (1 mg/mL, 500 μL). The mixture was gently tumbled at 4 °C for 1 h, and then concentrated in a Microsep concentrator (10 kDa MW cutoff) by centrifugation at 14 000g for 40 min at 4 °C. The concentrated biotinylated galectin-3 was brought up to 500 μL in phosphate saline buffer (PBS, Na_2HPO_4/NaH_2PO_4, 10 mM; NaCl, 150 mM, pH 7.2) and stored below 4 °C until use. Matrix-assisted laser desorption/ionization mass spectrometry showed an average incorporation of two biotin molecules per molecule of galectin-3.

Two microscale columns with identical column volumes (19.6 μL) were prepared by packing controlled-pore glass beads covalently coupled to streptavidin (CPG-SA) into orange PEEK tubings (ID, 0.5 mm; length, 100 mm). One column was saturated with *d*-biotin (1 mg/mL in PBS buffer) and served as a blank column for control experiments. The other column was saturated with biotinylated galectin-3 in PBS buffer (0.3 mg/mL) by infusion at flow rate of 8 μL/min for 125 min. Any unoccupied biotin binding sites on the streptavidin were blocked by infusion of *d*-biotin, and the column was washed with PBS buffer and kept refrigerated at 4 °C for later use.

Assay. The FAC/MS apparatus has been previously described.[14] The system has three 1 mL syringes in parallel on a multisyringe pump (PHD 2000, Harvard Apparatus) with a switching valve (Rheodyne, Model 9735) connected to an inlet of the immobilized galectin-3 column. The column outlet is connected to a tee for addition of makeup solvent that flows directly into a Hewlett-Packard 1100 MSD single quadruple electrospray mass spectrometer. Three syringes contained test samples, ammonium acetate buffer (NH_4OAc, 2 mM; NaCl, 0.2 mM, pH 7.2), and the makeup solution (CH_3CN), respectively. All solutions were infused simultaneously with the syringe pump at a flow rate of 8 μL/min per syringe. The column effluent from the sample was combined with the makeup flow in the tee to give a total flow rate of 16 μL/min

entering into the mass spectrometer. After each run, the column was reequilibrated with buffer by switching the loading valve. For characterization of the eluent, the spectrometer was set to scan from *m*/*z* 100 to 1500 in 1.5 s in the positive-ion mode. For screening of mixtures, the spectrometer was operated in selected-ion monitoring (locked on the chamber voltage of −3500 with a grounded electrospray needle, a N_2 drying gas flow rate of 4 L/min, and a N_2 nebulizer pressure of 480 mbar were used). Breakthrough volumes were measured at midpoints in the extracted ion chromatograms. All data were processed with Microsoft Excel software.

K_d determinations of **1** and **9** were conducted in duplicate with a range of concentrations between 2.4 and 20 μM.

Competitive inhibition of **9** was demonstrated using the following procedure. First, the retention time of **1** (3 μM) was determined. The column was washed out completely and then saturated by a solution of **9** (3 μM). Without washing the column, **1** (3 μM) was infused again to check whether the retention time had changed.

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