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Synthesis and Evaluation of New Thiodigalactoside-Based Chemical Probes to Label Galectin-3

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New chemical probes were synthesized to label galectin-3. They are based on the high affinity thiodigalactoside ligand. The probes were synthesized with benzophenone or acetophenone moieties as the photolabel for covalent attachment to the protein. Besides labeling the protein, these aromatic photolabels also greatly enhance the affinity of the probes towards galectin-3, due to the interaction of the photolabel with two arginine guanidinium groups of the protein. The linkage between the sugar and the photolabel was varied as an ester, an amide, and a triazole. For the amide and triazole derivatives, a versatile synthetic route towards a symmetrical 3-azido-3-deoxy-thiodigalactoside was developed. The new probes were evaluated for their binding affinity of human galectin-3. They were subsequently tested for their labeling efficiency, as well as specificity in the presence of a protein mixture and a human cancer cell lysate.

Introduction

The galectins are a family of β-galactoside-binding proteins.[1–3] Fifteen mammalian galectins have been identified and they can be subdivided into those containing one or two carbohydrate-binding domains.[2–4] The galectins are involved in many different biological processes, including apoptosis,[5–8] inflammation,[9–14] cell signaling,[7,15,16] cell adhesion,[17] and cancer progression.[16] Galectins are present intra- and extracellularly, and their expression is altered in tumor cells compared to normal, healthy cells.[18] A change in subcellular localization has been observed during the transition from normal cells to cancer cells.[15,18] Galectins interact with a number of intracellular proteins that are involved in cell regulation—for example galectin-3 interacts with oncogenic Ras,[19,20] cell cycle regulators such as the cyclins, and cell cycle inhibitors such as p21 and p27.[21–23] In several tumor cell types, it was shown that galectin-3 possesses anti-apoptotic activity.[6,15,24,25] Selective galectin-3 inhibitors have potential as (indirect) inducers of apoptosis,[26] but also as anti-inflammatory agents.[27] Because the mechanisms in which galectin-3 plays a role are generally poorly understood, a synthetic molecular probe that can detect and identify certain galectins would be particularly useful in generating a better understanding of the role of this protein, both in and outside the cell. We previously reported on the chemical probe-based method.[27–30] In this paper we used the best available galectin-3 inhibitors,[31] based on thiodigalactoside, as the starting point for a new series of galectin-3 probes. This class of compounds is interesting because of their high affinity, stability, and their ease of synthesis. Free thiodigalactoside has a K_d of 43 μM. Attachment of an aromatic moiety to the 3-OH of the sugar increases the affinity 10–1000 times;[31] this is due to a favorable interaction of an aromatic ring of the ligand with the side chains of Arg144 and 186.[32–35] By attaching aromatic photolabels to the 3-OH of the probe, we take advantage of this phenomenon. Various photolabels are used in the literature,[36,37] however, the aryl ketones are reported to have the highest labeling specificity and efficiency.[38] Another advantage of this type of photolabel is that UV irradiation can be used with light of wavelengths of over 300 nm, and this greatly limits the damage to the protein. The position of the photolabel with respect to nearby amino acid residues of the protein in the bound state is critical for efficient labeling. This is one of the reasons why some variations in the linker between the photolabel and the sugar moiety were made. The linker can also affect affinity, specificity, and sensitivity to proteolytic degradation. Our studies started with the preparation of a series of ester derivatives of thiodigalactoside **1**.[39,40] After evaluation of these derivatives (**7**–**9**), the less hydrophobic acetophenone,[41] was also incorporated (**10**). Despite the greater loss in entropy upon binding, the flexibility of a photolabel can highly improve the labeling efficiency.[42] We subsequently synthesized probes with amide (**16**, **17**) and triazole (**19**) linkers to further increase the affinity for galectin-3. The amide linkage was part of the best known galectin-3 ligands,[31] and triazole moieties are known to mimic amide

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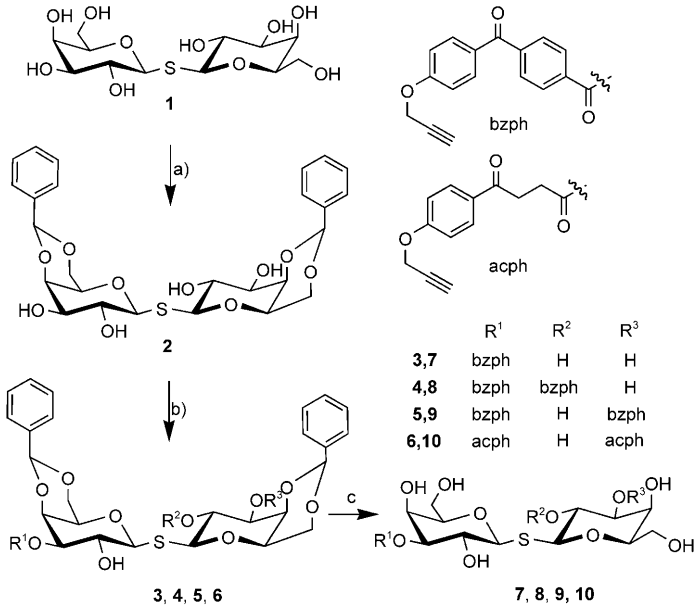
bonds.[43] Furthermore, a triazole moiety is known to enhance affinities for certain galectins.[44–46] To enable these syntheses a new 3-azido-3-deoxy-thiodigalactoside building block was prepared that allowed easy access to different amide and triazole derivatives.

Results and Discussion

Synthesis of ester-linked probes

The synthesis of thiodigalactoside was performed as previously described.[39] To couple the photolabel selectively to the 3-position of the sugar, ideally the other free hydroxyl groups had to be protected. However, as recently shown,[40] it is possible to use only a benzylidene protecting group on the 4- and 6-positions of the thiodigalactoside. Coupling takes place preferentially at the 3-position, and this obviates the need to protect the 2-OH. Still, minor coupling to the 2-OH occurred as well (Scheme 1). For the benzophenone-based probes the 3-mono-



	R^1	R^2	R^3
3,7	bzph	H	H
4,8	bzph	bzph	H
5,9	bzph	H	bzph
6,10	acph	H	acph

Scheme 1. Synthesis of ester-linked photoprobes. A) benzaldehyde dimethyl acetal (4.5 equiv), camphor sulfonic acid (CSA) (0.1 equiv), DMSO, 60 °C, 20 h, 68%; B) HOBt-ester (3 equiv), Et_3N, DCM, 2 h, 19–51%; c) AcOH/H_2O 3:1 (*v/v*), 90 °C, 1 h, 32–67% for **5** and **6**, 14% for **4**, 87% for **3** (when using 1.5 equiv of HOBt-ester).

substituted, and 2,3′-disubstituted compounds (**3** and **4**) were actually isolated and evaluated. The use of *N*-hydroxybenzotriazole esters to couple the photoprobes proved more efficient than coupling of the corresponding *N*-hydroxysuccinimide (NHS) esters. It brought reaction times down from 18 to about 2 hours, and selectivities appeared to be similar. After deprotection of the coupled products, probes **7**–**10** were obtained.

Synthesis of the amide- and triazole-linked probes

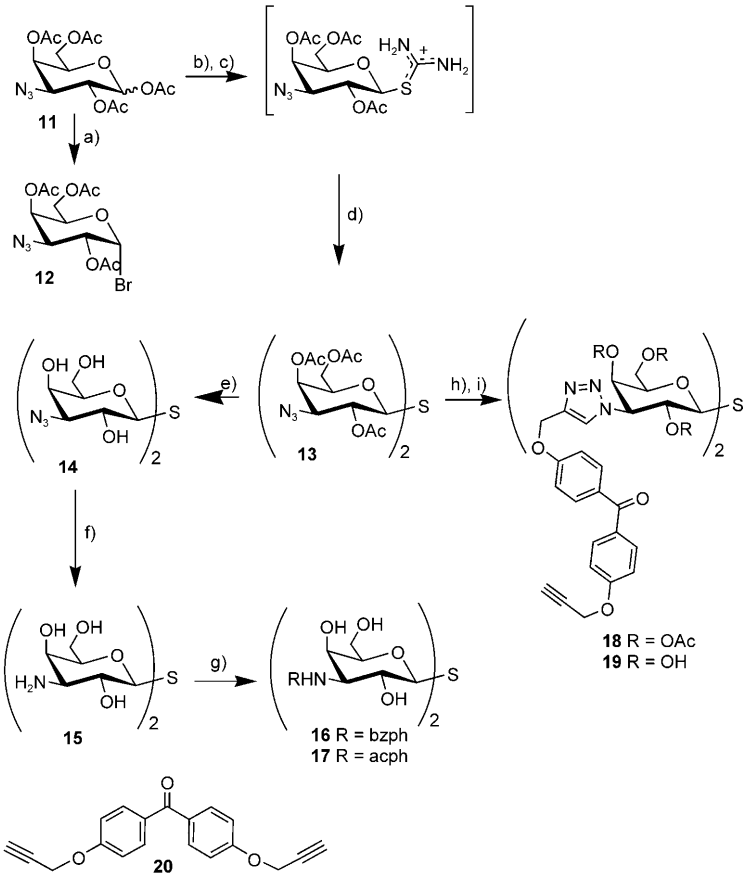
The amide- and triazole-linked probes can either be made by coupling two monosaccharide building blocks, each containing a photolabel, or by first synthesizing the appropriate thiodigalactoside, and subsequently attaching the photolabel. The advantage of the second strategy is its flexibility, because multiple labels can be made from a common intermediate. First the azido-galactose building block **11** (Scheme 2) was synthesized according to known procedures.[47–50] However, a conventional sodium sulfide coupling to the thiodigalactoside did not work with this compound.[33] After some experimentation, the use of thiourea, as used for the preparation of thioglycosides, proved successful.[51–53] Compound **11** was treated with $BF_3{\cdot}Et_2O$ and two equivalents thiourea. Subsequent addition of **12**, together with an excess of base, resulted in the formation of **13** as the main product. The difficult purification resulted in a low isolated yield of 37%, although the product is present in the reaction mixture in at least 70% (as judged by TLC).

For the synthesis of the amide-linked derivatives, the azido groups on the disaccharide were reduced by the Staudinger reaction[54,55] using PPh_3, which was more efficient than catalytic hydrogenation. The subsequent coupling reaction of the diamine with activated esters of the photolabels proved problematic, likely due to acetyl migration to the nitrogen.[56] To overcome this, the hydroxyl groups of **13** were first deprotected, followed by Staudinger reduction of the azides to give **15**, and the coupling of the photolabels through their NHS esters. Only low yields of coupling products were obtained; this was due to the limited solubility of the compounds, which complicated their purification. Finally, probes **16** and **17** were obtained after Zemplen deprotection. The triazole-linked probe **19** was prepared using a click chemistry reaction of **13** and the photolabel-linked dialkyne **20** followed by removal of the acetyl groups. In the click reaction the photolabel was added in a tenfold excess with respect to the sugar, to prevent polymerization.

Binding affinity of the probes for galectin-3

First, all probes were tested for binding to human galectin-3 with a fluorescence polarization assay.[57] The results are shown in Table 1. When we compare the probes to *N*-acetyl lactosamine derivative **21** (Galβ1-4GlcNAc β-OMe) or thiodigalactoside **1**, all of the 3,3′-substituted sugars (**9**, **10**, **16**, **17**, **19**) show increased affinity. The mono- (**7**) and 2,3′-disubstituted (**8**) analogues lose affinity compared to the unsubstituted **1**. The observation for **8** can be rationalized by computer modeling studies. It is clear that a substitution on the 2-OH either hinders the binding of the sugar to the protein, or, alternatively, it unfavorably points into the solvent. Furthermore, the 2-OH can no longer contribute as a hydrogen bond donor if it is substituted, and finally the photoprobe on the 2 position cannot reach either of the two crucial arginine residues to enhance the binding.

When we compare the benzophenone-linked (**9**, **16**) probes with the acetophenone-linked ones (**10**, **17**), for both ester and



Scheme 2. Synthesis of the amide- and triazole- linked photoprobes A) $TiBr_4$, CH_2Cl_2/EtOAc (10:1 *v/v*) 3 d, 48%; B) $BF_3{\cdot}Et_2O$, CH_3CN, 5 min, 0 °C; C) thiourea, CH_3CN, 80 °C, 2 h; D) **12**, Et_3N, 4 h, 34%; E) NaOMe, MeOH, 1 h, quant.; F) PPh_3, CH_3CN/H_2O, 20 h, quant.; G) OSu ester (3 equiv), CH_3CN/0.1 M $NaHCO_3$, 20 h, 8–18%; H) 10 equiv **20**, 0.15 equiv $CuSO_4$, 0.3 equiv sodium ascorbate, DMF, 20 min, 80 °C, μwave; I) NaOMe, MeOH, Dowex H^+, quant. over two steps.

to enhance the affinity—that is, the flexible acetophenone moiety and the amide linkage, and showed a K_d of 0.9 μM.

Molecular modeling

In order to evaluate to what extent the binding affinities can be predicted/explained by molecular methods we used the Lamarckian genetic algorithm in Autodock 4.[58] The scoring function was previously tested with 188 PDB complexes with success and had a standard error of around 1.5 log units.[59] The free energy estimate includes intermolecular and intramolecular energies from bound and unbound conformations, as well as solvation parameters for atoms and entropic penalties for torsional freedom. We used the local docking procedure as implemented in Yasara,[60] which means minimization followed by rigid docking. The method was tested with the redocking of the high-affinity cyclic urea ligand DMP323, binding to HIV-protease (PDB ID: 1mes) and found two clusters of results with a K_d of 295 pM and 965 pM as the best cluster hits. This is remarkably close to the experimental K_d value of 0.5 nM.[61] Furthermore, the redocking results for LacNAc and LacNAc 3′-tetrafluoro-*p*-OMe-benzamide binding to galectin-3, as given in Table 2, also suggest that the scoring function is remarkably accurate.

Two X-ray structures were used in the modeling study: 1) 1kjl in which LacNAc is bound to galectin-3, and 2) 1kjr in which LacNAc 3′tetrafluoro-*p*-OMe-benzamide is bound, a ligand that benefits from interactions of its 3′ aromatic group with Arg144. The difference between these structures is the open position

Table 1. K_d values of all the probes for galectin-3.[a]

Compound	K_d [μM]	Relative affinity
thiodigal (**1**)	49	1
7	143	0.3
8	>250	<0.2
9	14	3.5
10	2	25
16	2.4	20
17	0.9	54
19	23	2.1
LacNAc-β-OMe (**21**)	59	0.8

[a] Values were determined by a fluorescence polarization assay. LacNAc-OMe **21** and thiodigalactoside **1** are included in the Table as reference compounds.[40]

Table 2. Results of computer docking.

Compound	Calcd K_i [μM] with 1kjl	Calcd K_i [μM] with 1kjr	Exp. K_d [μM][a]
LacNAc	88.0 (RMSD 0.95 Å)[c]	57.3	59[b]
LacNAc 3′-tetrafluoro-*p*-OMe-benzamide	75.8	4 (RMSD 1.1 Å)[c]	0.88[35]
9	0.14	0.18	14
10	102	35	2
17	80	28	0.9
19	0.14	1.1	23

[a] Values were determined by fluorescence polarization. [b] for the anomeric β-OMe derivative. [c] deviation of the modeled ligand position from that in the crystal structure.

amide linkages, the acetophenone probes showed improved affinity. When comparing the linkages in the benzophenone series, the triazole-linked compound **19** was significantly less effective than the ester- (**9**) and amide- (**16**) linked versions. The best compound (**17**) contained both the optimal elements of Arg144 in 1kjr; this allows it to interact with the aromatic ring. Cross-docking of these ligands to the other PDB file shows that LacNAc 3′-tetrafluoro-*p*-OMe-benzamide has indeed a strong preference for the galectin-3 conformation in PDB ID: 1kjr, in which Arg144 is in a more open conformation to allow

for interaction with the aromatic ring. This can be seen in Table 2, in which its predicted affinities are 4 μM and 75.8 μM for 1kjr and is1kjl, respectively.

In our modeling procedure the ligand and protein side chains within 7 Ångstrom of the ligand are subjected to minimization and simulated annealing with the Amber force field before rigid (re)docking within Autodock. Following this procedure, the structure of the complex of the original ligand LacNAc and galectin-3 revealed only minor changes to the ligand and the protein when compared to 1kjl. During modeling of the compounds **9**, **10**, **17**, and **19** only Arg168 moves towards the ligands. Next, modeling studies were performed with the protein structure part of 1kjr. These studies also revealed only minor changes to the structure of galectin-3. Arg144 moves slightly and Arg168 more pronounced during the modeling of compounds **9**, **10**, **17**, and **19**. Furthermore, modeling predicts the bis-triazole compound **19** to be of very high affinity, and interact with Arg144, 162, 168 and 186 as well as with Trp181 and Glu184 (Figure 1).

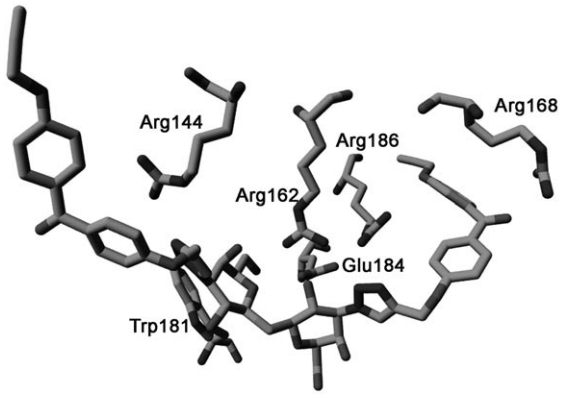


Figure 1. Modeling structure of the complex of 19 and galectin-3 (derived from 1kjl). Selected residues in direct contact with the ligand are shown.

Although the predicted K_i values are nicely within the range of the experimental values, the rank ordering is quite different. Compound **19** was measured and found to be the worst probe, whereas it is predicted to be the best. Surprisingly, in the modeling of the series of probes with the 1kjr-derived structures with exposed arginine, most probes actually bound worse than they do to the 1kjl-derived structure. (Table 2)

Galectin-3 labeling experiments

In order to see if the probes indeed were able to label galectin-3, they were exposed to the previously reported protocol.[30] In this protocol the protein and the probe are incubated, exposed to UV radiation and subsequently a fluorescein azide moiety is attached to the probe's alkyne group by using click chemistry. The labeling was visualized by running the protein samples on an SDS-PAGE gel and visualizing fluorescent proteins in a fluorescent scanner. In order to confirm that the synthesized probes indeed bind in the binding pocket of human galectin-3, probe **7** and isomeric probes **8** and **9** were also incubated in the presence of varying amounts of competing lactose, a relatively weak ligand for galectin-3 ($K_d \approx 0.2$ mM) (Figure 2). In all cases labeling of galectin-3 was observed, but this was much better for the probes containing two photolabels, despite a tenfold lower concentration. However, only in the case of the 3,3′-disubstituted thiodigalactoside **9** competition by lactose was observed. The observation for **9** seems to be in agreement with the measured K_d value, considering that the labeling does not go to zero since the gel reflects the accumulation of covalent bound probe over time as previously noted.[30] No competition was observed for **7** and **8** and these compounds had high K_d values for galectin-3; this indicates that these compounds label nonspecifically.

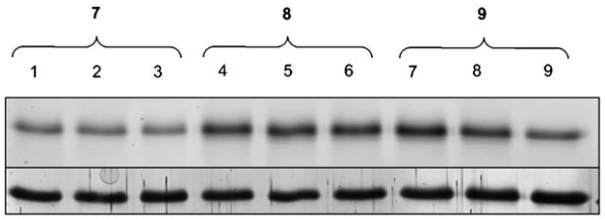


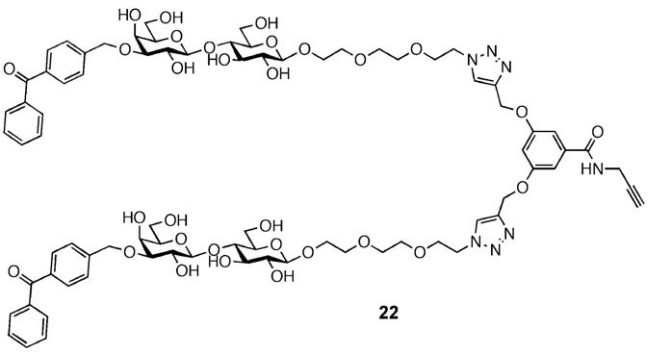
Figure 2. Comparison of galectin-3 labeling by probes **7**, **8**, and **9**, with 30 ng of human galectin-3 per sample and different concentrations of lactose. Lane 1–3: **7** (10 μM) and 0, 1, and 10 mM lactose; Lanes 4–6: **8** (1 μM) and 0, 1, and 10 mM lactose; Lanes 7–9: **9** (1 μM) and 0, 1, and 10 mM of lactose. Upper panel: fluorescence picture, lower panel: silver stain shows equal protein loading.

Labeling specificity of the probes

Compound **9** was also compared to the previously reported[30] divalent probe **22** ($K_d = 4$ μM) for their specificity in binding galectin-3 in the presence of other proteins. Both probes were incubated with 10, 50 or 100 ng of galectin-3 and five other proteins (100 ng each; Figure 3). From this fluorescent picture it seems that both probes are comparable in their selectivity for binding galectin-3. However, a tenfold lower concentration of **9** can be used which is indicative of an enhanced labeling efficiency.

A direct comparison was made with the other probes for their ability to label galectin-3. Figure 4A shows their respective labeling efficiencies. Strikingly, the acetophenone-based probes **10** and **17** showed the most pronounced labeling. This is likely due to the stronger interaction between the probe and galectin-3, and/or the increased mobility of the photolabel. Apparently, these properties are more important than the reactivity (see the Supporting Information) of the photolabel itself. Interestingly, **19** seems to label galectin-3 efficiently at lower concentrations.

Based on the above results probes **10**, **17**, and **19** were chosen for an experiment in which the probes are compared for their ability to label human galectin-3 specifically in the presence of many other proteins. A human cancer (HeLa) cell lysate was used as a relevant protein environment. The result is shown in Figure 4B. Both the acetophenone-probes **10** and



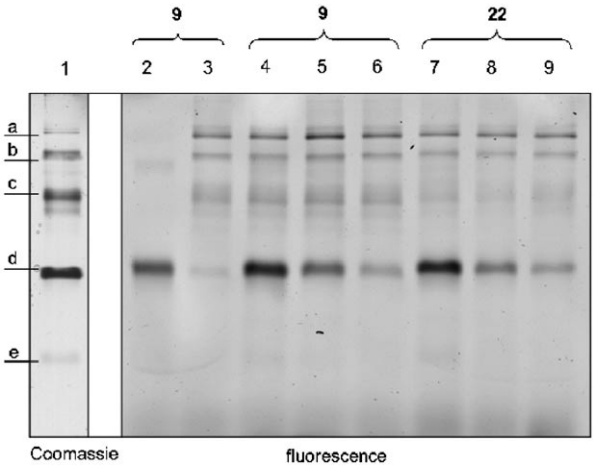


Figure 3. Comparison of **9** with the divalent probe **22**, for their specificity for galectin-3 labeling in a mixture of other proteins. In all samples, 100 ng of all the proteins is present, except for lane 2. These proteins are (from high to low molecular weight): A) phosphorylase B, B) albumin, C) ovalalbumin, D) carbonic anhydrase and E) trypsin inhibitor. The amount of galectin-3 was varied over the samples. Lane 1: Coomassie stain of lane 3; Lane 2: 10 ng galectin-3, labeled with 1 μM of **9**; Lane 3: protein mixture without any galectin-3 present, labeled with 1 μM of **9**; Lanes 4–6: 1 μM of **9** with 100, 50, 10 ng galectin-3 respectively; Lane 7–9: 10 μM of divalent probe **22** with 100, 50 and 10 ng galectin-3 respectively.

17 were able to label galectin-3. However when increasing amounts of other proteins were added, the probes seemed to have a preference for labeling the highly abundant proteins present in the cell lysate. Surprisingly, this is in contrast with the results observed for the benzophenone-triazole probe **19**. Even with up to 2 μg of other proteins, the probe still labeled its target protein specifically.

Conclusions

New chemical probes have been synthesized to label galectin-3 and they were evaluated in the presence of other proteins and in a human cancer cell lysate. Three different linkages between the disaccharide and the photolabel were compared in binding affinity, labeling efficiency, and labeling specificity of galectin-3. Also two different photolabels were compared. A new disaccharide building block was synthesized that allowed a rapid assembly of a novel thiodigalactoside-triazole probe. Taking all the different experiments together, it can be concluded that firstly, the labeling efficiency and affinity is higher for the acetophenone-derivatives than for the benzophenone-derivatives. The linker between the acetophenone and the sugar is more flexible; this may allow a more optimal stacking of an aryl moiety onto Arg144 and 186 of the protein, thereby increasing the affinity as well as labeling efficiency over the more rigid, benzophenone-based probes. As for the affinity, however, the opposite trend was obtained from the computer modeling, although the calculated affinities were within the range of the experimental values. Secondly, the amide derivatives have a higher binding affinity for galectin-3 than the ester derivatives, with a factor of two and six. The affinity improvements obtained in this study are mainly reflected in the concentrations of the chemical probe that can be used in the biochemical labeling experiments. Unfortunately, being able to use the probes at lower concentrations did not improve their selectivity for the target protein. Finding an effective photoprobe is a multidimensional challenge with issues such as affin-

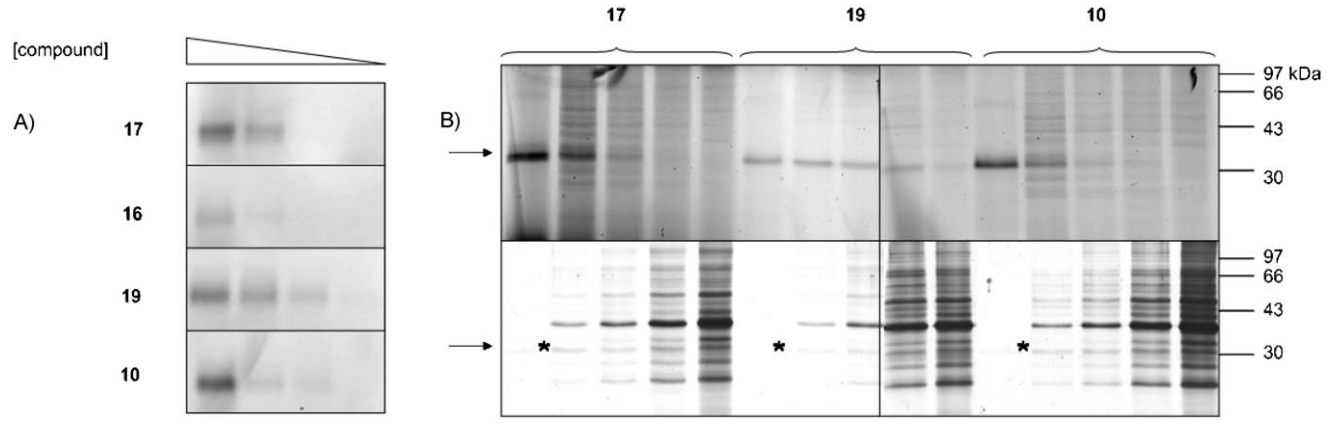


Figure 4. A) Concentration ranges of the probes show the comparison in the efficiency of labeling galectin-3. 50 ng of galectin-3 is present in each sample. Concentrations of the probes are (from left to right) 10, 1, 0.5, and 0.1 μM respectively. B) Comparison of the thiodigalactoside based probes in labeling human galectin-3 in the presence of a human cancer (HeLa) cell lysate. In all the lanes, 50 ng of galectin-3 is present. Concentration ranges were made of the cell lysate: from left to right for each probe 0, 0.25, 0.5, 1, and 2 μg of total protein lane. Probe concentrations: **17**: 2 μM; **10**: 2 μM; **19**: 1 μM. Upper panel: fluorescence picture, lower panel: silver stain of the corresponding lanes (arrows and asterisks denote the position of galectin-3).

ity, labeling efficiency, lipophilicity, and flexibility that all impact the finale outcome in a complex protein mixture. These issues may similarly play a role in drug efficacy in a complex protein environment. In a labeling experiment flexibility of the photoprobe can increase the labeling efficiency but rigidity is better if the geometry is of the probe is appropriate.[42] Notably, rigidity seems beneficial for optimal selectivity in complex mixtures as seen for **19**, which was really specific for its target protein. Affinity played only a secondary role, as this compound only exhibited limited affinity for galectin-3, although the computer docking predicted a very high affinity. The triazole linkage in probe **19** is a very attractive attribute of this compound and possibly crucial for its favorable properties. Gratifyingly, its synthesis was also the most straightforward. Additional advantages of the triazole moiety over the ester and amide include its stability towards chemical hydrolysis, reduction, oxidation and proteases.[46] Probe **19** greatly contributed to solving the nonspecific binding issue in galectin-3 labeling about which we reported previously.[30] The probe may find applications in proteomics and cell biology experiments, and thereby provide an additional tool in the study towards the roles of galectin-3.

Experimental Section

General methods: All reagents were purchased from commercial sources and used without further purification. Dowex 50×8 (H^+ form, 20–50 mesh) was purchased from Sigma. Analytical thin layer chromatography (TLC) was performed on Merck precoated silica gel 60 F254 (0.25 mm) plates. Spots were visualized with UV light, H_2SO_4, or ninhydrin. Column chromatography was carried out with Merck Kieselgel 60 (40–63 mm). ^{1}H NMR and ^{13}C NMR were obtained on a Varian 300 MHz spectrometer. Chemical shifts are given in ppm with respect to internal TMS for ^{1}H NMR. ^{13}C NMR spectra were recorded using the attached proton test (APT) pulse sequence. Two-dimensional ^{1}H,^{1}H correlation and total correlation spectroscopy (COSY and TOCSY) and ^{1}H,^{13}C correlated heteronuclear single quantum coherence (HSQC) NMR spectra (500 MHz) were recorded at 300 K with a Varian Unity INOVA 500 spectrometer. Low resolution ESI-MS experiments were performed on a Shimadzu LCMS QP8000 system. Exact masses were measured by nanoelectrospray time-of-flight mass spectrometry on a Micromass LCToF mass spectrometer at a resolution of 5000 FWHM. Gold-coated capillaries were loaded with 1 μL of sample (concentration 20 μM) dissolved in CH_3CN/H_2O (1:1, *v/v*). NaI or poly(ethylene glycol) (PEG) was added as internal standard. The capillary voltage was set between 1100 and 1350 V, and the cone voltage was set at 30 V.

4,4′,6,6′-*O*-Benzylidene-1-thio-β-D-digalactopyranoside (2):[40] Thiodigalactoside **1** (948 mg, 2.65 mmol) was dissolved in DMSO (16 mL). Camphor sulfonic acid (CSA, 62 mg, 0.3 mmol) and benzaldehyde dimethyl acetal (1.79 mL, 11.9 mmol) were added at intervals. The mixture was stirred at 60 °C for 20 h. The reaction was quenched with a few drops of NEt_3, and concentrated in vacuo. The product (**2**) was purified by using silica gel column chromatography and obtained as a white solid (960 mg, 68% yield). R_f = 0.50 (CH_2Cl_2/MeOH 9:1, *v/v*) ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.51 (s, 1H; 5-H), 3.59 (dd, $J_{2,3}$ = 3.0 Hz, $J_{1,2}$ = 9.5 Hz, 1H; 2-H), 4.01 (d, $J_{a,b}$ = 12.5 Hz, 1H; 6a-H), 4.20 (d, $J_{3,2}$ = 3.0 Hz, 1H; 3-H), 4.29 (d, $J_{a,b}$ = 12.5 Hz, 1H; 6b-H), 3.70 (d, $J_{1,2}$ = 9.5 Hz, 1H; 1-H), 4.40 (d, $J_{4,3}$ = 9.5 Hz, 1H; 4-H), 5.50 (s, 1H; C*H*C_6H_5), 7.21–7.41 (m, 10H; 10H_{arom}); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 69.4, 70.1, 71.3, 73.6, 76.1 (C-6, C-4, C-5, C-2, C-3), 85.5 (C-1), 101.3 (*C*HC_6H_5), 126.5–129.4 (10CH_{arom}), 137.5 (2C_{arom}).

General procedure for the synthesis of a HOBt-esters: Benzophenone and acetophenone derivatives **24** and **26** (see Supporting Information) (0.5 mmol) and HOBt·H_2O (2.0 mmol) were dissolved in dry THF (10 mL) and stirred under N_2 for about 20 min. 4-Dimethylaminopyridine (DMAP, 0.15 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 2.0 mmol) were added to the mixture and the mixture was stirred for 1 h. The mixture was decanted onto a small silica plug. Flash chromatography was performed in hexane/EtOAc (1:1 *v/v*), and the first fractions were pooled and concentrated in vacuo. The white solid that was obtained was used immediately in the synthesis of **3**–**6**.

3,3′-Dibenzophenone-4,4′,6,6′-*O*-benzylidene-1-thio-β-D-digalactopyranoside (5): Compound **2** (61 mg, 0.11 mmol) was dissolved in dry CH_2Cl_2 (2 mL). The HOBt-ester (3 equiv) and NEt_3 (70 μL) were added. The reaction was stirred for 2 h, after which it was diluted with CH_2Cl_2 (50 mL), washed with sat. $NaHCO_3$ (2×25 mL), dried with Na_2SO_4, filtered, and concentrated in vacuo. The product **5** was obtained from silica column chromatography (hexane/EtOAc 1:1→1:2, *v/v*) in 51% yield as a white solid (59 mg, along with 16 mg or 14% of **4**, see below). R_f = 0.66 (CH_2Cl_2/MeOH 92:8, *v/v*); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.56 (s, 1H; CH_2CCH), 3.77 (s, 1H; 5-H), 4.13 (d, $J_{a,b}$ = 12.5 Hz, 1H; 6a-H), 4.38 (d, $J_{a,b}$ = 12.5 Hz, 1H; 6b-H), 4.60 (d, $J_{3,2}$ = 9.5 Hz, 1H; 3-H), 4.71 (s, 1H; 1-H), 4.76 (s, 2H; OCH_2CCH), 5.05 (dd ≈ t, $J_{2,3}$ = 9.5 Hz, 1H; 2-H), 5.18 (d, $J_{4,3}$ = 10.0 Hz, 1H; 4-H), 5.51 (s, 1H; C*H*C_6H_5), 6.81–7.01 (m, 10H; 10$H_{benzylidene}$), 7.50 (d, J = 8.0 Hz, 2H; $COC_3H_2C_3H_2OCH_2CHCH$), 7.53 (d, J = 8.0 Hz, 2H; $COC_3H_2C_3H_2COO$), 7.72 (d, J = 8.5 Hz, 2H; $COC_3H_2C_3H_2OCH_2CHCH$), 7.97 (d, J = 8.5 Hz, 2H; $COC_3H_2C_3H_2COO$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 56.1 (OCH_2CCH), 67.8, 69.7, 71.8, 75.0, 75.9 (C-2, C-6, C-5, C-3, C-4), 76.5 (CH_2CCH), 86.4 (C-1), 103.1 (*C*HC_6H_5), 114.7 ($HCCCH_2OCC_2H_2C_2H_2CCO$), 127.1, 128.2, 129.5 (10$CH_{benzylidene}$), 136.9 (2$C_{arom,\ benzylidene}$), 129.1 ($COCC_2H_2C_2H_2CCOO$), 130.2 ($COCC_2H_2C_2H_2CCOO$), 130.4 ($HCCCH_2OCC_2H_2C_2H_2CCO$), 132.3 ($HCCCH_2OCC_4H_4CCO$), 132.7 ($COCC_4H_4CCOO$), 142.2 ($COCC_4H_4CCOO$), 161.5 ($HCCCH_2OCC_4H_4CCO$), 166.2 ($COCC_4H_4CCOO$), 194.8 ($C_{arom}COC_{arom}$).

2,3′-Dibenzophenone-4,4′,6,6′-*O*-benzylidene-1-thio-β-D-digalactopyranoside (4): The product was obtained from the above reaction as a white solid (14% yield, 16 mg). R_f = 0.72 (CH_2Cl_2/MeOH 92:8, *v/v*); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.56 (s, 2H; $2CH_2CCH$), 3.69 (s, 2H; 2×5-H), 4.06 (d, $J_{a,b}$ = 12.6 Hz, 2H; 2×6a-H), 4.07–4.16 (m, 1H; 2b-H), 4.20 (t, J = 8.8 Hz, 1H; 3b-H), 4.36–4.41 (m, 3H; 2×6b-H, 2a-H), 4.54 (dd, $J_{3,4}$ = 3.5 Hz, $J_{3,2}$ = 12 Hz, 1H; 3a-H), 4.60 (d, $J_{1,2}$ = 7.0 Hz, 2H; 2×1-H), 4.77 (s, 4H; OCH_2CCH), 5.18 (dd, $J_{4,3}$ = 3.5 Hz, $J_{4,5}$ = 9.2 Hz, 2H; 4-H), 5.53 (s, 1H; CH_AC6H5), 5.63 (s, 1H; $CH_BC_6H_5$), 7.04 (d, J = 8.5 Hz, 4H; $COC_3H_2C_3H_2OCH_2CHCH$), 7.19–7.49 (m, 10H; 10$CH_{benzylidene}$), 7.71–7.83 (m, 8H; $CH_{2arom\ A,B}COCH_{2arom\ A,B}$), 8.09 (d, J = 8 Hz, 2H; $CH_{2,\ arom,\ B}COO$), 8.20 (d, J = 8 Hz, 2H; $CH_{2,arom,A}COO$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 56.1 (OCH_2CCH), 64.6, 68.2 (C-6), 69.4 (C-3b), 70.0 (C-5), 73.4 (C-2b), 74.5 (C-3a), 75.3, 76.0, 76.6 (CH_2CCH), 79.8, 84.7 (C-2a), 86.0 (C-1), 101.4 (CHC_6H_5-a), 103.4 (*C*HC_6H_5-b), 114.9 ($HCCCH_2OCC_2H_2C_2H_2CCO$), 126.3, 128.5, 129.7 (10$CH_{benzylidene}$), 137.5 (2$C_{arom,\ benzylidene}$), 129.2 ($COCC_2H_2C_2H_2CCOO$), 130.4 ($COCC_2H_2C_2H_2CCOO$), 130.4 ($HCCCH_2OCC_2H_2C_2H_2CCO$), 132.3 ($HCCCH_2OCC_4H_4CCO$), 132.7 ($COCC_4H_4CCOO$), 138.9, 142.5 ($COCC_4H_4CCOO$), 161.6 ($HCCCH_2OCC_4H_4CCO$), 165.9 ($COCC_4H_4CCOO$), 194.8 ($C_{arom}COC_{arom}$).

3,3′-Dibenzophenone-1-thio-β-D-digalactopyranoside (9): Compound **5** (59 mg) was dissolved in acetic acid/H_2O (3:1, *v/v*) and refluxed for 1 h at 90 °C. Disappearance of the starting material was followed by TLC analysis. The solvent was coevaporated with MeOH (3×) in vacuo, and the product purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1, *v/v*) and obtained as a white solid (38% yield, 19 mg). R_f = 0.66 (CH_2Cl_2/MeOH 8:2, *v/v*); ^{1}H NMR (300 MHz, CD_3OD): δ = 3.02 (t, J = 2 Hz, 2H; C≡C*H*), 3.61-3.95 (m, 6H; H-5, H-6ab), 4.06 (t, $J_{2,3}$ = 9.6 Hz, 2H; H-2), 4.23 (d, $J_{4,3}$ = 3.3 Hz, 2H; H-4), 4.59 (~s, 2H; H-1), 5.09 (dd, $J_{3,2}$ = 9.6 Hz, $J_{3,4}$ = 3.3 Hz, 2H; H-3), 7.12 (d, J = 9.0 Hz, 4H; CH_{ar}), 7.80 (d, J = 8.1 Hz, 4H; CH_{ar}), 7.81 (d, J = 9.0 Hz, 4H; CH_{ar}), 8.25 (d, J = 8.1 Hz, 4H); HRMS (*m/z*): calcd for $C_{46}H_{42}O_{16}SNa$ $[M+Na]^+$ 905.2086; found 905.2080.

2,3′-Dibenzophenone-1-thio-β-D-digalactopyranoside (8): Compound 4 (16 mg, 15 μmol) was dissolved in acetic acid/H_2O (3:1, *v/v*, 5 mL) and heated for 1 h at 90 °C. Disappearance of the starting material was followed by TLC analysis. The solvent was coevaporated with MeOH (3×) in vacuo, and the product was purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1, *v/v*) and obtained as a white solid (67% yield, 9 mg, 10 μmol). R_f (CH_2Cl_2/MeOH 9:1, *v/v*) = 0.14; ^{1}H NMR (300 MHz, CD_3OD): δ = 2.98, 3.02 (2t, J = 2.4 Hz, 2H; C≡C*H*), 3.38–3.42 (m, 2H; H-5), 3.55–3.77 (m, 4H; H-6ab), 3.95–4.08 (m, 4H; H-2, H-4), 4.49–4.74 (m, 4H; H-1, H-3), 7.11 (~t, J = 9.0 Hz, J = 8.1 Hz, 4H; CH_{ar}), 7.74–7.86 (m, 8H; CH_{ar}), 8.14 (d, J = 8.1 Hz, 2H; CH_{ar}), 8.24 (d, J = 8.7 Hz, 2H; CH_{ar}); HRMS (*m/z*): calcd for $C_{46}H_{43}O_{16}SNa$ $[M+Na]^+$ 905.2086; found 905.2065.

3-Benzophenone-4,4′,6,6′-*O*-benzylidene-1-thio-β-D-digalactopyranoside (3): Compound **2** (64 mg, 0.12 mmol) was dissolved in dry CH_2Cl_2 (2 mL). The HOBt-ester (1.5 equiv) and Et_3N (70 μL) were added. The reaction was stirred for 2 h, after which it was diluted with CH_2Cl_2 (50 mL), washed with saturated $NaHCO_3$ (2×25 mL), dried with Na_2SO_4, filtered and concentrated in vacuo. The product was obtained from silica column chromatography (hexane/EtOAc 2:1→3:1, *v/v*) as a white solid (87% yield, 82 mg). R_f = 0.42 (CH_2Cl_2/MeOH 92:8, *v/v*); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.57 (t, J = 2 Hz, 1H; C≡CH), 3.56 (~s, 1H; H-5′), 3.68 (dd ~m, 1H; H-2′), 3.75 (~s, 1H; H-5), 4.05–4.13 (m, 2H; H-6b, H-6b′), 4.25 (d, $J_{6a',6b'}$ = 3.5 Hz, 1H; H-6a′), 4.30 (d, $J_{1',2'}$ = 12.5 Hz, 1H; H-1′), 4.37–4.40 (m, 2H; H-4′, H-6a), 4.51–4.58 (m, $J_{1,2}$ = 9.5 Hz, 2H; H-1, H-3′), 4.69 (d, $J_{4,3}$ 3.0 Hz, 1H; H-4), 4.78 (d, J = 2 Hz, 2H; HC≡CC*H*$_2$O), 4.93 (t, $J_{2,3}$ = 10.0 Hz, $J_{2,1}$ = 9.5 Hz, 1H; H-2), 5.13 (dd, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 10.0 Hz, 1H; H-3), 5.46 (s, 1H; C*H*C′$_{benzylidene}$), 5.58 (s, 1H; C*H*C$_{benzylidene}$), 6.95–7.13 (m, 6H; $CH_{benzylidene}$), 7.03 (d, J = 9.0 Hz, 2H; $CH_{benzoph}$), 7.37 (d, J = 7 Hz, 2H; CH′$_{benzylidene}$), 7.49 (d, J = 7.5 Hz, 2H; $CH_{benzylidene}$), 7.54 (d, J = 8.5 Hz, 2H; $CH_{benzoph}$), 7.76 (d, J = 8.5 Hz, 2H; $CH_{benzoph}$), 7.90 (d, J = 8.0 Hz, 2H; $CH_{benzoph}$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 56.1 (OCH$_2$C≡CH), 67.5, 69.4, 69.8, 70.5, 71.4, 71.9, 73.9, 74.9, 75.9, 76.3, 76.5 (C-2, C-2′, C-3, C-3′, C-4, C-4′, C-5, C-5′, C-6, C-6′, C≡*C*H, *C*≡CH), 85.8 (C-1′), 86.2 (C-1), 101.8 (*C*HC′$_{benzylidene}$), 102.7 (*C*HC$_{benzylidene}$), 114.8 (2x *C*$_{benzoph}$COCH$_2$C≡C), 126.7- 132.7 (18x C_{ar}), 137.2 (2x CHC$_{benzylidene}$), 142.1 (COO), 161.6 (*C*$_{benzoph}$OCH$_2$C≡C), 166.3 (*C*$_{benzoph}$COO), 194.9 (C=O$_{benzoph}$).

3-Benzophenone-1-thio-β-D-digalactopyranoside (7): Compound **3** (16 mg, 20 μmol) was dissolved in acetic acid/H_2O (3:1 *v/v*, 5 mL) and heated for 1 h at 90 °C. Disappearance of the starting material was followed by TLC analysis. The solvent was coevaporated with toluene (3×) in vacuo, and the product purified by silica gel column chromatography (CH_2Cl_2/MeOH 92:8, *v/v*) and obtained as a white solid (32% yield, 4 mg, 6 μmol). R_f (CH_2Cl_2/MeOH 8:2, *v/v*) = 0.11; ^{1}H NMR (300 MHz, CD_3OD): δ = 3.03 (t, J = 2.4 Hz, 1H; C≡C*H*), 3.46–3.99 (m, 10H; H-2, H-4, H-5, H-6ab), 4.48 (dd, $J_{3',4'}$ = 4.2 Hz, $J_{3',2'}$ = 11.7 Hz, 1H; H-3′), 4.61 (dd, $J_{3,4}$ = 3 Hz, $J_{3,2}$ = 9 Hz, 1H; H-3), 4.70 (d, $J_{1',2}$ = 9.6 Hz, 1H; H-1′), 4.75 (d, $J_{1,2}$ = 9.6 Hz, 1H; H-1), 7.14 (d, J = 9.0 Hz, 2H; CH_{ar}), 7.80–7.85 (m, 4H; CH_{ar}), 8.22 (d, J = 8.4 Hz, 2H; CH_{ar}); HRMS (*m/z*): calcd for $C_{29}H_{32}O_{13}SNa$ $[M+Na]^+$ 643.1456; found 643.1469.

3,3′-Diacetophenone-4,4′,6,6′-*O*-benzylidene-1-thio-β-D-digalactopyranoside (6): Compound **2** (59 mg, 0.11 mmol) was dissolved in dry CH_2Cl_2 (2 mL). The HOBt-ester (3 equiv) and NEt_3 (70 μL) were added. The reaction was stirred for 2 h, after which it was diluted with CH_2Cl_2 (50 mL), washed with saturated $NaHCO_3$ (2× 25 mL), dried with Na_2SO_4, filtered and concentrated in vacuo. The product was obtained from silica column chromatography (hexane/EtOAc 1:2, *v/v*) as a white solid (19% yield, 20 mg, 20 μmol). R_f = 0.49 (CH_2Cl_2/MeOH 92:8, *v/v*); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.56 (t, J = 2 Hz, 2H; C≡CH), 2.70 (t, J = 6.9 Hz, 4H; C*H*$_2$COO), 3.15 (t, J = 6.9 Hz, 4H; CH$_2$C*H*$_2$COC$_{ar}$), 3.58 (s, 1H; H-5) 4.01 (d, $J_{a,b}$ = 13 Hz, 2H; H-6a), 4.26 (d, $J_{a,b}$ = 13 Hz, 1H; H-6b), 4.40 (d, $J_{4,3}$ = 3 Hz, 2H; H-4), 4.48 (d, $J_{1,2}$ = 9.6 Hz, 2H; H-1), 4.73 (m, $J_{OCH2C\equiv CH}$ = 2 Hz, 6H; OC*H*$_2$C≡CH, H-2), 4.99 (dd, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 3.3 Hz, 2H; H-3), 5.46 (s, 2H; C*H*C$_6$H$_5$), 6.94 (d, $J_{acetoph}$ = 9.0 Hz, 4H; CH_{ar}), 7.06–7.46 (m, 10H; $CH_{benzylidene}$), 7.85 (d, $J_{acetoph}$ = 9.0 Hz, 4H; CH_{ar}); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 28.8 (*C*H$_2$COO), 33.4 (CH$_2$*C*H$_2$COC$_{ar}$), 56.0 (OCH$_2$C≡CH), 67.8, 69.5, 71.4, 74.5, 74.9 (C-2, C-3, C-4, C-5, C-6), 76.4 (C≡*C*H), 78.0 (*C*≡CH), 85.9 (C-1), 102.4 (*C*HC$_{benzylidene}$), 114.7 (2C$_{acetoph}$), 127.1, 128.1, 128.4, 129.1, 130.5 (8C_{ar}), 137.1 (CHC$_{benzylidene}$), 161.5 (CH$_2$OC$_{acetoph}$), 173.2 (C(=O)O), 196.8 (*C*=O).

3,3′-Diacetophenone-1-thio-β-D-digalactopyranoside (10): Compound **6** (20 mg, 20 μmol) was dissolved in acetic acid/H_2O 3:1 *v/v* (5 mL) and refluxed for 1 h at 90 °C. Disappearance of the starting material was followed by TLC analysis. The solvent was coevaporated with MeOH (3×) in vacuo, and the product purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1, *v/v*) and obtained as a white solid (50% yield, 8 mg, 10 μmol). R_f = 0.15 (CH_2Cl_2/MeOH 9:1, *v/v*); HRMS (*m/z*): calcd for $C_{38}H_{42}O_{16}SNa$ $[M+Na]^+$ 809.2086; found 809.2092.

2,4,6-Tri-*O*-acetyl-3-azido-3-deoxy-α-D-galactopyranosyl bromide (12): Compound **11** (637 mg, 1.69 mmol) was dissolved in CH_2Cl_2/EtOAc (10:1, 44 mL). Titanium tetrabromide (1.11 g, 2.5 mmol) was added and the mixture was stirred for three days. The reaction was quenched by adding NaOAc (1 g) and stirring for 1 h. The suspension was diluted with CH_2Cl_2 and extracted with water. The organic layer was dried with Na_2SO_4, filtered and evaporated. **12** was purified from the residue with silica gel column chromatography (hexane/EtOAc 3:1) and isolated as a yellowish oil (48% yield, 317 mg, 0.80 mmol); R_f = 0.42 (hexane/EtOAc 3:1); ^{1}H NMR ($CDCl_3$): δ = 2.07, 2.16, 1.18 (3 s, 9H; OC*H*$_3$), 4.03 (dd, $J_{6a,5}$ = 6.9 Hz, $J_{6a,6b}$ = 11 Hz, 1H; H-6a), 4.12 (dd, $J_{3,4}$ = 3.3 Hz, $J_{3,2}$ = 10.5 Hz, 1H; H-3), 4.17 (dd, $J_{6b,5}$ = 6 Hz, $J_{6a,6b}$ = 11 Hz, 1H; H-6b), 4.40 (t, $J_{5,6}$ = 6 Hz, 1H; H-5), 4.93 (dd, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 3.9 Hz, 1H; H-2), 5.48 (dd, $J_{4,3}$ = 3.3 Hz, $J_{4,5}$ = 1 Hz, 1H; H-4), 6.69 (d, $J_{1,2}$ = 3.9 Hz, 1H; H-1); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 28.2, 28.3, 28.4 (3OC(O)*C*H$_3$), 68.7 (C-6), 66.1, 74.9, 77.2, 79.1 (C-2, C-3, C-4, C-5), 96.1 (C-1), 177.3, 177.5, 177.9 (3C=O).

3,3′-Diazido-3,3′-dideoxy-2,2′,4,4′,6,6′-hexa-*O*-acetyl-β-D-thiodigalactoside (13): Compound **11** was dissolved in dry CH_3CN (1 mL) and stirred on an ice bath. $BF_3\cdot Et_2O$ (74 mL) was added and the mixture was stirred for 5 min at 0 °C. Thiourea (61 mg, 0.8 mmol) was added and the mixture was refluxed for 2 h. The mixture was allowed to cool to RT. Bromide **12** (238 mg, 0.60 mmol) was dissolved in dry CH_3CN (1 mL) and added to the mixture. NEt_3 (0.5 mL) was added and the mixture was stirred for 20 h. The mix-

ture was diluted with CH_2Cl_2 (100 mL) and washed with H_2O (1×) and brine (1×). The organic layer was dried with Na_2SO_4, filtered and evaporated. **13** was purified with silica gel column chromatography (hexane/EtOAc/CH_3CN 300:80:20→200:85:15, *v/v/v*) and obtained in 34% yield. R_f = 0.30 (hexane/EtOAc 1:1); ^{1}H NMR ($CDCl_3$): δ = 2.06, 2.13, 2.17 (3 s, 9H; OCH_3), 3.64 (dd, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 3.3 Hz, 1H; H-3), 3.84 (dt, $J_{5,6}$ = 6.6 Hz, $J_{5,4}$ = 1.2 Hz, 1H; H-5), 4.11 (d, $J_{6.5}$ = 6.6 Hz, 2H; H-6ab), 4.78 (d, $J_{1,2}$ = 9.9 Hz, 1H; H-1), 5.17 (t, $J_{2,1}$ = 9.9 Hz, 1H; H-2), 5.47 (dd, $J_{4,5}$ = 1.2 Hz, $J_{4,3}$ = 3.3 Hz, 1H; H-4); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 20.9, 21.0 (3 OC(O)*C*H3), 61.7 (C-6), 63.1, 67.9, 68.6, 75.7 (C-2, C-3, C-4, C-5), 81.4 (C-1), 169.6, 170.1, 170.6 (3 C=O). ESI-MS: *m/z* 683.2 $[M+Na]^+$.

3,3′-Diazido-3,3′-dideoxy-β-D-thiodigalactoside (14): Compound **13** (14 mg, 0.022 mmol) was dissolved in MeOH (10 mL). A few drops of a NaOMe-solution (30% *w/v* in MeOH) were added and the mixture was stirred for 3 h. The mixture was neutralized with Dowex H^+ resin, filtered, and concentrated in vacuo. The colorless oil was obtained in quantitative yield (10 mg, 0.022 mmol). R_f = 0.79 (EtOAc/MeOH/H_2O 4:2:1); ^{1}H NMR (300 MHz, CD_3OD): δ = 3.36 (dd, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 3.0 Hz, 2H; H-3), 3.57 (dt, $J_{5,4}$ = 1 Hz, $J_{5,6a}$ = 7.5 Hz, $J_{5,6b}$ = 4.5 Hz, 2H; H-5), 3.64 (dd, $J_{6b,5}$ = 4.5 Hz, $J_{6b,6a}$ = 11.4 Hz, 2H; H-6b), 3.74 (dd, $J_{6a,5}$ = 7.5 Hz, $J_{6a,6b}$ = 11.4 Hz, 2H; H-6a), 3.84 (t, *J* = 9.9 Hz, 2H; H-2), 3.94 (dd ~ d, $J_{4,3}$ = 3.0 Hz, 2H; H-4), 4.71 (d, $J_{1,2}$ = 9.9 Hz, 2H; H-1); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 61.5, 67.2, 68.5, 68.9, 80.0, 84.5.

3,3′-Diamino-3,3′-dideoxy-β-D-thiodigalactoside (15): Compound **14** (0.066 mmol) was suspended in CH_3CN (3 mL). PPh_3 (44 mg, 0.17 mmol) was added. When the PPh_3 was dissolved completely, H_2O (1 mL) was added to dissolve the sugar. The clear reaction mixture was stirred for 20 h. TLC analysis (EtOAc/MeOH/H_2O 4:2:1) showed the product on the baseline. The mixture was diluted with H_2O (30 mL) and washed twice with EtOAc. The H_2O-layer was evaporated in vacuo and a yellow solid was obtained in quantitative yield (23 mg, 0.066 mmol). ^{1}H NMR (300 MHz, D_2O): δ = 2.86 (dd, $J_{3,2}$ = 9.9 Hz, $J_{3,4}$ = 2.6 Hz, 2H; H-3), 3.40 (t, *J* = 9.9 Hz, 2H; H-2), 3.60 (m, 6H; H-5, H-6a, H-6b), 3.85 (dd ~ s, 2H; H-4), 4.70 (d, $J_{1,2}$ = 9.9 Hz, 2H; H-1); ^{13}C NMR (75.5 MHz, D_2O): δ = 56.8, 61.4, 67.8, 69.6, 80.1, 84.2. ESI-MS: *m/z* 356.95 $[M+H]^+$.

3,3′-Dideoxy-3,3′-(dibenzophenone-amide)-β-D-thiodigalactoside (16): Compound **15** was dissolved in $NaHCO_3$ solution (1 mL, 0.1 M). Compound **24** was dissolved in CH_3CN (1 mL). The two solutions were mixed together with DMF (1 mL) to dissolve the reagents completely. The mixture was stirred for 20 h. The mixture was concentrated in vacuo and the product purified using silica gel column chromatography (5→15% MeOH in CH_2Cl_2). After lyophilization, a white powder was obtained (6.2 mg, 7.0 μmol, 18%). R_f = 0.62 (MeOH/CH_2Cl_2 2:8). ^{1}H NMR (500 MHz, DMSO): δ = 2.54 (t, *J* = 6.5 Hz, 4H; CH_2C(=O)NH), 3.17 (t, *J* = 6.5 Hz, 4H; CH_2C(=O)O), 3.53–3.54 (m, 4H; H-6ab), 3.65 (s, 2H; C≡C*H*), 3.73 (dd, $J_{2,1}$ = 10 Hz, $J_{2,3}$ = 16.5 Hz, 2H; H-2), 4.00 (ddd, 2H; H-3), 4.62 (t, $J_{5,6ab}$ = 5.5 Hz, 2H; H-5), 4.75 (d, $J_{1,2}$ = 10 Hz, 2H; H-1), 4.92–4.97 (m, 6H; H-4, CH_2O), 7.16 (d, *J* = 9.0 Hz, 4H; CH_{ar}), 7.77 (dd, 8H; CH_{ar}), 8.07 (d, *J* = 8.5 Hz, 4H; CH_{ar}NH), 8.31 (d, *J* = 7.5 Hz, 2H; NH); HRMS (*m/z*): calcd for $C_{46}H_{44}N_2O_{14}SNa$: 903.2405 $[M+Na]^+$; found 903.2391.

3,3′-Dideoxy-3,3′-(diacetophenone-amide)-β-D-thiodigalactoside (17): Compound **15** (15 mg, 42 μmol) and OSu ester **27** (41 mg, 125 μmol, see supporting information) were suspended in CH_3CN (2 mL) and a $NaHCO_3$ solution (0.1 M, 1 mL) was added. The pH was checked (≈8) and the mixture was stirred for 20 h. The mixture was concentrated in vacuo and **17** was isolated using preparative HPLC. After lyophilization, a white powder was obtained (8% yield, 2.7 mg, 3.4 μmol). R_f = 0.093 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (500 MHz, DMSO): δ = 2.50–2.57 (t, 4H; CH_2C(O)Ar), 3.18 (t, *J* = 7 Hz, 4H; CH_2C(O)NH), 3.33–3.40 (m, 4H; H-6a, H-6b), 3.46 (dd, 2H; H-2), 3.62 (t ~ s, 2H; C≡C*H*), 3.71 (ddd, 2H; H-3), 4.57 (t, $J_{5,6ab}$ = 6 Hz, 2H; H-5), 4.61 (d, $J_{1,2}$ = 9.5 Hz, 2H; H-1), 4.78 (d, $J_{4,3}$ = 5.5 Hz, 2H), 4.91 (d, *J* = 2 Hz, 4H; OCH_2), 7.09 (d, *J* = 9 Hz, 4H; CH_{ar}), 7.76 (d, *J* = 7.5 Hz, 2H; NH), 7.96 (d, *J* = 9 Hz, 4H; CH_{ar}). ESI-MS: *m/z* 785.80 $[M+H]^+$; HRMS (*m/z*): calcd for $C_{38}H_{45}N_2O_{14}SNa$: 807.2405 $[M+Na]^+$; found 807.2403.

3,3′-(Ditriazol-4-benzophenone-1-yl)-2,2′,4,4′,6,6′-hexa-*O*-acetyl β-D-thiodigalactoside (18): Compound **13** (5.2 mg, 7.8 μmol), sodium ascorbate (0.5 mg, 2.3 μmol), $CuSO_4$ (0.3 mg, 1.2 μmol) and OSu ester **25** (22 mg, 0.075 mmol, see Supporting Information) were dissolved in DMF (2 mL). The mixture was heated under microwave irradiation to 80 °C for 20 min. The mixture was concentrated in vacuo and the product purified with silica gel column chromatography (MeOH/CH_2Cl_2 2:98). The product was obtained as a white solid in quantitative yield. R_f = 0.48 (CH_2Cl_2/MeOH 9:1, *v/v*). ^{1}H NMR ($CDCl_3$): δ = 1.87, 1.95, 2.07 (3 s, 6H; OCH_3), 2.56 (t, *J* = 2.4 Hz, 2H; C≡C*H*), 4.08–4.22 (m, 6H; H-5, H-6a, H-6b), 4.77 (d, *J* = 2.4 Hz, 4H; $OCH_2CC{\equiv}CH$), 4.98 (d, $J_{1,2}$ = 9.6 Hz, 2H; H-1), 5.16 (dd, $J_{3,2}$ = 11.1 Hz, $J_{3,4}$ = 3.3 Hz, 2H; 3-H), 5.28 (s, 2H; OCH_2(triazole)), 5.57 (dd ~ d, $J_{4,3}$ = 3 Hz, 2H; H-4), 5.68 (t, $J_{2,1}$ = 9.6 Hz, $J_{2,3}$ = 11.1 Hz, 1H; H-2), 6.99 (d, *J* = 8.4 Hz, 2H; CH_{ar}), 7.04 (d, *J* = 8.7 Hz, 2H; CH_{ar}) 7.67 (s, 1H; C=C$H_{triazole}$), 7.77 (d, *J* = 8.4 Hz, 2H; CH_{ar}), 7.79 (d, *J* = 8.7 Hz, 2H; CH_{ar}); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 20.4, 20.7, 20.9 (3 C(O)CH_3), 56.1 ($OCH_2C{\equiv}CH$), 61.5 ($OCH_2C_{triazole}$), 62.2 (C-6), 63.3, 66.6, 68.7, 75.9 (C-2, C-3, C-4, C-5), 76.4 (*C*≡CH), 82.4 (C-1), 114.5, 114.6 (4 CH_{ar}), 131.5 (2 C_{ar}), 132.4, 132.5 (2 CH_{ar}), 144.5 (N-*C*=C), 161.0, 161.3 (2 C_{ar}), 168.9, 169.6, 170.6 (3 *C*(O)CH_3), 194.4 (C=O).

3,3′-(Ditriazol-4-benzophenone-1-yl)-β-D-thiodigalactoside (19): 18 (7.8 μmol) was dissolved in MeOH (10 mL), and a few drops of a 30% (*w/v*) NaOMe-solution in MeOH were added. The mixture was stirred for 4 h, neutralized with Dowex H^+ resin, filtered, and the solvent was evaporated in vacuo. The white solid was dissolved in a few mL CH_3CN/H_2O (1:1, *v/v*) and lyophilized to obtain a white powder (9 mg) in quantitative yield. ^{1}H NMR (300 MHz, CD_3OD): δ = 3.00 (t, *J* = 2.4 Hz, 2H; C≡CH), 3.67 (dd, $J_{3,2}$ = 10 Hz, $J_{3,4}$ = 3 Hz, 2H; H-3), 3.75–3.84 (m, 6H; H-5, H-6ab), 4.10 (d, $J_{4,3}$ = 3 Hz, 2H; H-4), 4.95 (t, 2H; H-2), 4.95 (d, $J_{1,2}$ = 10 Hz, 2H; H-1), 5.29 (s, 4H; $OCH_2C_{triazole}$), 7.12 (~dd, *J* = 9 Hz, 8H; CH_{ar}), 7.75 (d, *J* = 9 Hz, 8H; CH_{ar}), 8.32 (s, 2H; NC*H*=CN), 8.54 (s, 2H; NH); HRMS (*m/z*): calcd for $C_{50}H_{48}N_6O_{14}SNa$ $[M+Na]^+$ 1011.2841; found 1011.2913.

Galectin-3 binding assay by fluorescence polarization: Galectin-3 was produced and potency of inhibitors determined by fluorescent polarization in principle as described.[57] A fluorescein tagged A-tetrasaccharide (0.1 μM, **4** in Table SII of Ref. [62]) was used as tracer (probe) with galectin-3 (1 μM), and compounds were tested as inhibitors using concentrations between ~0.5 and 500 μM, to cover a factor of at least ~5 below and above the concentration giving 50% inhibition.

Modeling: The 3D coordinates of galectin-3 with *N*-acetyllactosamine (lacNAc) and the fluorinated 3′-substituted carbohydrate were downloaded from the PDB (IDs: 1kjl and 1kjr). The difference between these structures is the open position of Arg144 in 1kjr; this open position allows hydrophobic rings to interact. The program Autodock 4.0[58] was used for the local docking experiments as implemented in Yasara. In this procedure the ligand and protein side chains within 7 Å are subjected to minimization with Amber before rigid docking with Autodock. To remove bumps and to correct the covalent geometry, the structure was energy-minimized

with the Amber99 force field,[63] using a 10.4 Å VdW force cutoff and the Particle Mesh Ewald algorithm[64] to treat long range electrostatic interactions. After removal of conformational stress by a short steepest descent minimization, the procedure was continued by simulated annealing (time step 2 fs, atom velocities scaled down by 0.9 every tenth step) until convergence was reached, that is, the energy improved by less than 0.1% during 200 steps (Yasara documentation). After that Autodock 4 was used to do a Solis and Wetts local search[58] with rigid protein and rigid ligand to find the best position and give an estimate of the free energy (K_i value) using a procedure described in.[59] The best local search parameters were empirically chosen to be 300 for the maximum amount of Solis and Wetts iterations, 15000 for the population size in the genetic algorithm and 200 local docking runs. These parameters always led to only a limited number of clusters (RMSD for clustering was taken to be 5.0 Å) and with the best hit well within 1.0 Angstrom from the X-ray complex during redocking of a test file (1 mes). The compounds **9**, **10**, **17**, and **19** were built in Yasara based on LacNAc in the 1JKL PDB file.

Typical procedure for the labeling of human galectin-3: One of the chemical probes (**7**, **8**, **9**, **10**, **16**, **17**, or **19**, with 1, 2 or 10 μL of a 50 μM stock solution in water/DMSO 10:1, or dilutions from this stock) was added to galectin-3 stock solution (3.8 μL, 0.1 mg mL^{-1} in HEPES buffer, human, recombinant form, carrier free, R&D Systems Europe, Abingdon, UK), together with a six protein mixture solution (10 μL, 200 μg mL^{-1} in each lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin and phosphorylase B), HeLa cell lysate (natural galectin-3 abundance about 5 ng per lane, based on a Western blot), or lactose, and the samples were adjusted to a total volume of 50 μL with HEPES buffer (pH 7.4, 150 mM NaCl). The resulting solutions were irradiated for 30 min under a 366 nm UV lamp at 4–5 cm distance at 4 °C. After photoincubation, ligand (6 μL, 100 μM in DMSO/*t*-butanol 1:4), $CuSO_4$ (2 μL of 50 mM in water), tris(2-carboxyethyl)phosphine (TCEP; 2 μL, 50 mM in water) and Alexa 488–N_3 conjugate (15 μL, 100 μM stock solution in water) were added and the volume adjusted to 100 μL with HEPES buffer. The samples were then gently shaken overnight in the dark at 4 °C and denatured (50 μL of a 10% SDS, 40% glycerol and 2% dithiothreitol solution by boiling at 95 °C for 5 min. From the total volume of 150 μL, 20 μL was loaded onto a Tris–HCl gel (15%) for SDS-PAGE. After extensive washing of the developed gel (1–5 h in water) to eliminate excess dye reagent, fluorescence was detected with a Typhoon fluorescence scanner and each gel was subsequently silver stained.

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