Journal of Molecular Catalysis B: Enzymatic 84 (2012) 108–114

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Chemo-enzymatic synthesis of functionalized oligomers of *N*-acetyllactosamine glycan derivatives and their immobilization on biomaterial surfaces

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ARTICLE INFO

Article history:
Available online 17 February 2012

Dedicated to Prof. Dr. Dr. h.c. Maria Regina Kula on the occasion of her 75th birthday (16th March 2012).

Keywords:
Poly-LacNAc
Glycosyltransferases
β1,4-galactosyltransferase
β1,3-*N*-acetyl-glucosaminyltransferase
Galectin

ABSTRACT

Poly-*N*-acetyllactosamines (poly-LacNAc, [−3Gal(β1–4)GlcNAc(β1−]$_n$) are terminal glycan structures present in glycoproteins and glycolipids. Their biological functions as ligands for galectins and as carriers of glycan epitopes are well documented. In the present paper we have characterized six novel functionalized β-D-GlcNAc derivatives, including aglyca of varying hydrophobicity and molecular weight, as substrates for recombinant human β1,4 galactosyltransferase 1 (β4GalT-1). The sugar derivatives carry short or long amino- or azide-terminated linker molecules for further modification or immobilization. The linker chemistry had an impact on enzyme kinetics and enzymatic syntheses of *N*-acetyllactosamine derivatives (LacNAc, Gal(β1–4)GlcNAc(β1-R). The combination of β4GalT-1 with bacterial β1,3-*N*-acetylglucosaminyltransferase (β3GlcNAc-T) resulted in the preparative syntheses of LacNAc oligomers with up to three LacNAc repeating units. All products were characterized by NMR and MS. The obtained LacNAc glycans were immobilized onto microtiter plates and their efficiency of binding of fungal galectin CGL2 was determined.

1. Introduction

The surface of eukaryotic cells universally carries a sugar coating consisting of glycoproteins and glycolipids. The carbohydrate moieties of these glycoproteins and glycolipids play an important role as information carrier system. One important sugar structure is *N*-acetyllactosamine (LacNAc type 2, Gal(β1–4)GlcNAcβ1-R) [1]. LacNAc and repeated units of LacNAc (poly-LacNAc, [3Gal(β1–4)GlcNAcβ1−]$_n$) represent a backbone for various modifications catalyzed by various glycosyltransferases; LacNAc type 2 is a basic structural element of terminal carbohydrate ligands like Lewis-x, Lewis-y, and blood groups [2]. Poly-LacNAc on the cell surface and on glycoproteins of the extracellular matrix (laminin, fibronectin) serves also as recognition structure and interaction partner for galectins, a class of carbohydrate-binding proteins which mediate cell–cell and cell–matrix interactions [3,4].

Glycan arrays were engineered to explore and understand the specificity of galectin binding [5–7]. A range of methods for stable covalent immobilization of glycans was developed, among them the Diels–Alder reaction [8], Cu-catalyzed azide-alkyne cycloaddition [9,10], and maleimide or *N*-hydroxysuccinimide mediated coupling [5,9]. These reactions require the appropriate chemical functionalization of carbohydrates at their reducing end to facilitate the desired chemical reaction with the functionalized solid support as well as chemical or enzymatic elongation steps [10]. In this context, chemo-enzymatic strategies have been developed for the synthesis of oligomers of LacNAc glycans [11–15]. We could further demonstrate that ECM glycoproteins can be cross-linked by a lectin onto immobilized oligo-LacNAc [13]. We are currently exploiting this approach for the development of an artificial extracellular matrix (ECM) on biomaterial surfaces for tissue engineering. However, enzymatic synthesis of functionalized glycans for subsequent immobilization on biomaterial surfaces depends strongly on the substrate promiscuity of the involved glycosyltransferases. Galactosyltransferase from bovine milk (bovine β4GalT, EC 2.4.1.22) has been studied extensively with regard to synthesis and substrate specificity [16,17]. However, only a few synthetic acceptor substrates were kinetically characterized for the conversion by bovine β4GalT [18] or recombinant human β4GalT [19].

In the present paper we demonstrate that the acceptor substrate spectrum of human placental β4GalT-1, expressed as the recombinant fusion protein His$_6$propeptide-catβ4GalT-1 (β4GalT-1) [19], can be significantly extended. With respect to the linker

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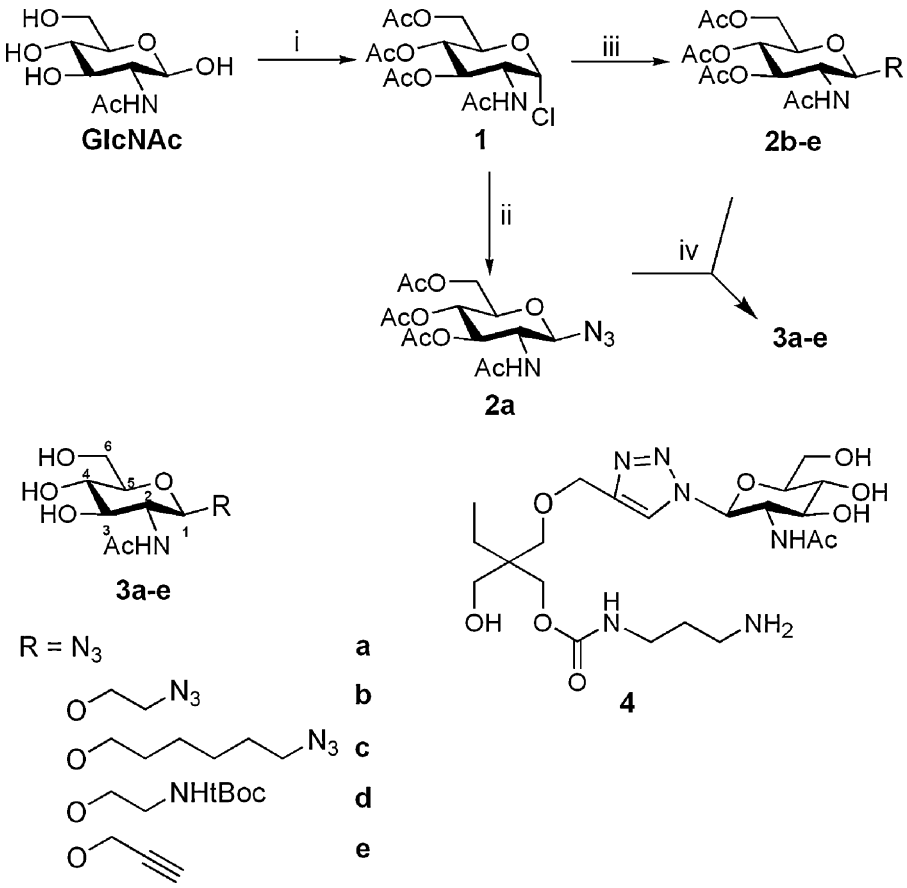
doi:10.1016/j.molcatb.2012.02.002



Scheme 1. Synthesis of β-*N*-acetylglucosaminides **3a–e** with (i) CH_3COCl, CH_2Cl_2, RT, 36 h; (ii) NaN_3, $(CH_3)_2C{=}O$, reflux, 16 h; (iii) alcohol of **b–e**, CH_2Cl_2, RT, 16 h; (iv) NaOMe, MeOH, 0 °C, 2 h.

aglycones, six novel substrates are characterized by donor- and acceptor kinetics. The series of tested substrates contain aglyca of varying hydrophobicity and molecular weight. GlcNAc modifications ranged from a very short azide functionalization to a saccharide-coupler structure **4**.

Based on kinetic data for β4GalT-1 efficient syntheses of LacNAc derivatives are achieved with quantitative yields. The LacNAc products are further elongated to yield LacNAc oligomers in a combinatorial one-pot synthesis employing three enzymes. Covalent immobilization of isolated LacNAc oligomers (up to LacNAc-trimer) onto microtiter plates are accomplished by the azide- or amino-terminated linkers for subsequent binding studies of the fungal galectin CGL2.

2. Experimental

2.1. Materials

Chemicals were purchased and used as follows: di-tert-butyl dicarbonate (Fluka >98%), silver perchlorate monohydrate (Alfa Aesar, 98%), silver carbonate (Applichem, pure), sodium azide (Merck), sodium ascorbate (Applichem), copper sulphate pentahydrate (Applichem), N-acetylglucosamine (Alfa Aesar 99%), propargyl alcohol (Acros Organics, 99%), acetyl chlorid (Acros Organics 99%), molybdatophosphoric acid hydrate (Merck), dichloromethane (DCM) (Kmf, p.a.), acetone (p.a. DHB Prolabo), chloroform (Fischer Scientific, HPLC grade), Celite545 (Roth), Kieselgel 60 (<0.2 mm) (Aplichem). Methanol was dried by refluxing over solid sodium and distillation. Water sensitive reactions were performed under inert gas (nitrogen). Activated donor saccharides were from Sigma–Aldrich. Lactate dehydrogenase, pyruvate kinase as well as anti-his6-peroxidase and diaminobenzidine substrate were from Roche. OPD substrate was from DakoCytomation, Denmark. All further chemicals were purchased from Roth.

2.2. Chemical syntheses

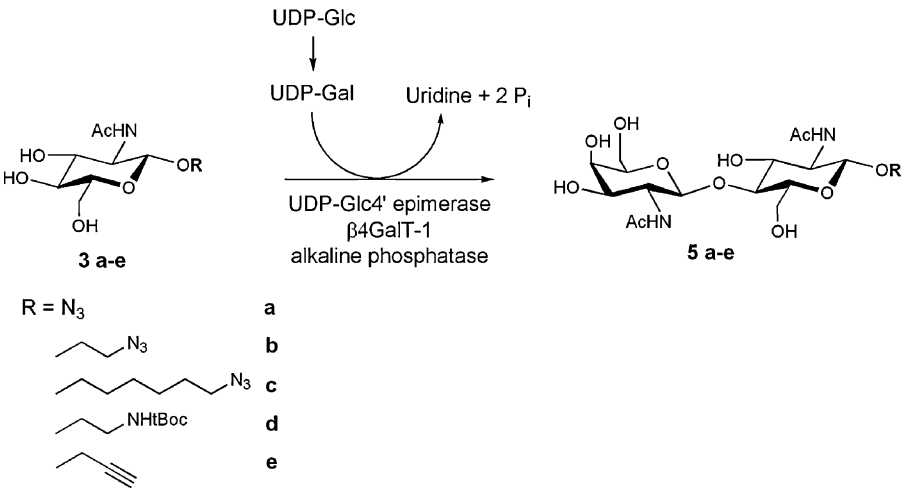
The chemical syntheses of acceptor substrates **3a–e** and **4** (Scheme 1) and their characterization were performed as described in supporting information.

2.3. Chromatography

Chemical reactions were monitored with thin-layer chromatography using pre coated Polygram R SIL G/UV254 (Macherey & Nagel, Düren, Germany). Spots were detected by UV light (254 nm). The sugar spots were visualized with 10% sulphuric acid in ethanol and heating with a heat gun. Column chromatography was performed on silica gel (Merck, corn size 0.063–0.200 mm).

2.4. NMR spectroscopy and mass spectrometry

^1H and ^{13}C NMR spectra were recorded with a Bruker DPX 300 for monosaccharides and Bruker AV 600 UltraShield™ (Bruker, Rheinstetten, Germany) for oligosaccharides at 298 °K in $CDCl_3$ or D_2O using *tetra*-methyl silane (^1H: $\delta = 0$ ppm; ^{13}C: $\delta = 0$ ppm) or residual signals from solvents ($CDCl_3$ ^1H: $\delta = 7.26$ ppm; ^{13}C: $\delta = 77.2$ ppm; D_2O ^1H: $\delta = 4.79$ ppm) as internal standards. ^1H peak



Scheme 2. Enzymatic synthesis of LacNAc derivatives (**5a**–**e**). UDP-Gal was generated by UDP-Glc 4′-epimerase (where indicated) and UDP from the reaction of β4GalT-1 with GlcNAc acceptors **3a**–**e** was hydrolyzed by alkaline phosphatase.

assignments were made by first order analysis of the spectra, supported by standard ^{1}H–^{1}H correlation spectroscopy (COSY). ^{13}C peak assignments were made by first order analysis of the spectra, supported by standard ^{1}H–^{13}C correlation spectroscopy (HMQC). Infrared (IR)-Spectroscopy was measured using a Nexus 470 FT-IR (ThermoNicolet, Wisconsin, USA).

Isolated products were analysed by HPLC/ESI mass spectrometry in the negative mode using a sample temperature of 400 °C; needle voltage of 4.5 kV; and cone voltage of 100 V. modus: negative. Samples of 10 μl of 0.1 mM glycan solution were concentrated on a Multospher 120 RP 18 HP-3 μ (60 mm × 2 mm) (CS-Chromatographie Service, Langerwehe, Germany), applying a flow rate of 0.2 mL/min using 50% acetonitrile in water as mobile phase.

2.5. Enzyme production and purification

Detailed protocols for the production, purification and assay of the recombinant enzymes used in this study were described previously for the human placental fusion protein His_6-propeptide-catβ4GalT-1 [13,19], the UDP-Glc/GlcNAc-4′-epimerase from *Campylobacter jejuni* [20,21], and β1,3-*N*-acetylglucosminyltransferase from *Helicobacter pylori* [13,22].

2.6. Kinetic characterization of products *3a*–*3e* and *4*

Kinetic experiments were performed according to Sauerzapfe et al. [19] by continuous photometric assay and discontinuous HPLC detection. Photometric microtiter plate assay in a total volume 250 μL contained 0.1 M ammonium acetate (pH 7.4), 25 mM KCl, 2 mM K_2HPO_4, 4 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.25 mM NADH + H^+, 2 mM $MnCl_2$, 5 U pyruvate kinase, and 5 U lactate dehydrogenase. The reaction was started by addition of 25 μL enzyme solution. The consumption of NADH + H^+ was followed at 340 nm at 37 °C. Control experiments for the detection of side activities were done without donor molecule and without enzyme solution. Acceptor kinetic measurements contained varying concentrations of acceptor substrates (0–10 mM of **3c** and **3d**, and **4**) with constant concentration of 5 mM (for **3a**–**3e**) or 3 mM UDP-Gal (for **3d**, **4**). Donor kinetic measurement was performed with varying concentrations of UDP-Gal (0–10 mM) and constant acceptor concentration of 3 mM for **3a**, **3b**, **3e**), 1 mM for **3d**, **4**, and 0.3 mM for **3c**. Kinetic constants were calculated according to appropriate kinetic equations by non-linear regression using Michalis–Menten equation $V = (V_{max} \times [S])/(K_m + [S])$ or the kinetic model for substrate inhibition calculation $V = V_{max}/(1 + (K_m/[S]) + ([S]/K_i))$. Data were fitted by Excel® Solver Algorithm and SigmaPlot 10 (SPSS Science Software GmbH, Erkrath, Germany).

2.7. Enzymatic syntheses

The enzymatic syntheses of the LacNAc products **5a**–**e** (Scheme 2) and the LacNAc oligomers **6c**–**9c** and **6d**–**9d** (Scheme 3) as well as their characterization are described in supporting information.

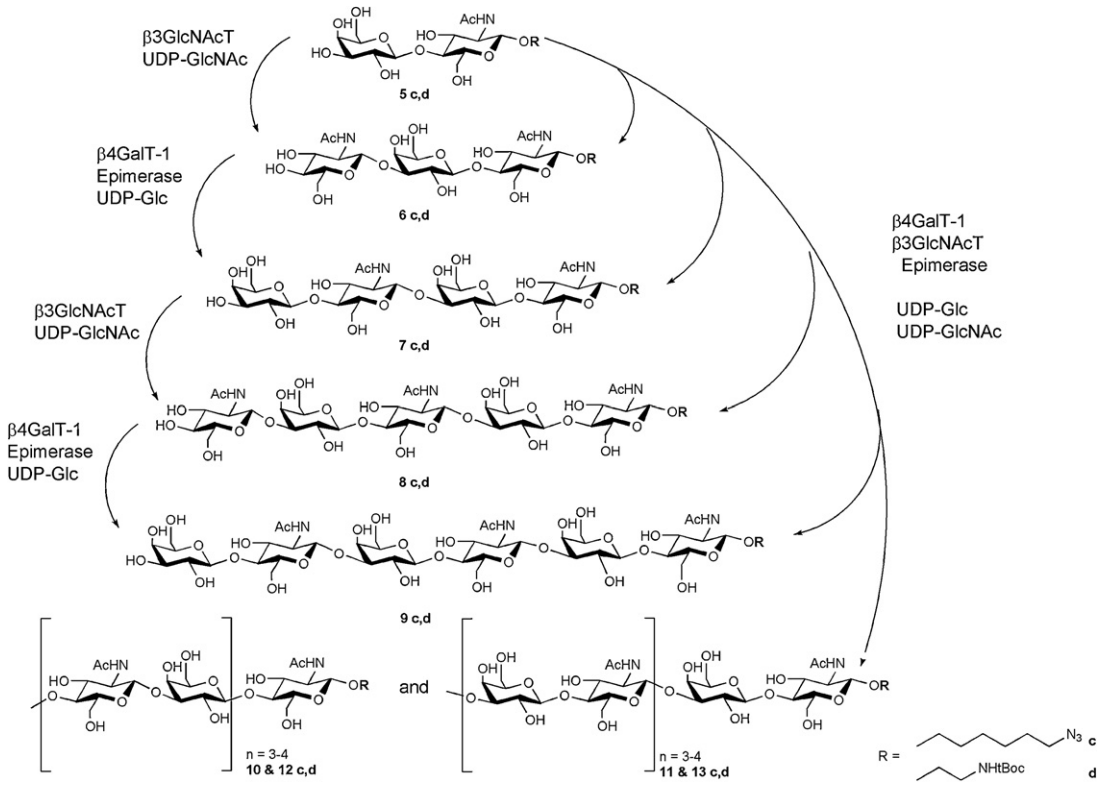
2.8. One-pot enzymatic synthesis, analysis and product isolation

One-pot synthesis (Scheme 3) was carried out in a total of 100 mL in buffer A (0.1 M ammonium acetate, pH 7.4 with 2.5 mM KCl, 1 mM DTT, 2 mM $MnCl_2$, 10 U alkaline phosphatase), 2 mM $MgCl_2$, 3.75 mM **5c** or **5d**, 7.5 mM UDP-GlcNAc, 15 mM UDP-Glc, His_6-propeptide-catβ4GalT-1 (0.8 U), β3GlcNAcT (0.4 U), UDP-Glc-4′-epimerase (0.6 U). The synthesis incubated for 24 h at 30 °C and 60 rpm in a glass beaker. Product conversion was observed by HPLC (LiChrospher® 100 RP18, LiChroCart (Merck), RT, 205 nm, 0,5 mL/min) with acetonitrile gradient (LacNAc oligomer-**c**: 5–30% (v/v) acetonitrile; LacNAc oligomer-**d**: 10–30% (v/v) acetonitrile). After 24 h the reaction was stopped by heating at 95 °C for 5 min. Denatured protein aggregates were removed by ultrafiltration for 60 min with VivaSpin20 MWCO 30,000 (VivaScience, Hannover, Germany). To gain also Gal-terminated structures His_6-propeptide-catβ4GalT-1 (0.4 U) and UDP-Glc-4′-epimerase (0.4 U) were added for further 24 h. Afterwards the reaction was stopped and filtrated. Semi-preparative HPLC (acetonitrile gradient as indicated above, 12 mL/min, 205 nm, Eurospher 100-10 C18, 250 mm × 20 mm, Knauer, Berlin, Germany,) enabled fractioning of defined LacNAc oligomer-**c** or **d** chain lengths as described above. The molecular mass of isolated products was confirmed by ESI-MS (Thermo Finnigan MSQ and Thermo Fischer Orbitrap XL) in negative mode as described above.

2.9. Immobilization and lectin assays

2.9.1. Click reaction in aminoreactive microtiter plates

To obtain an alkyne functionalized surface 40 mM propargylamine was incubated in 50 mM carbonate buffer pH 9.6 over night in aminoreactive microtiter plates (Nunc Immobilizer™, Nunc



Scheme 3. Sequential and one-pot preparative enzymatic synthesis of LacNAc oligomers **6c**,**d**–**9c**,**d**.

GmbH, Wiesbaden, Germany) at room temperature. The plate was washed three times with carbonate buffer afterwards. Click reaction in microtiter plate was performed as follows: 25 μL solution A contained 4 mM of saccharide (**5c**–**9c**) in H_2O, 25 μL solution B contained 2 mM $CuSO_4$, and 8 mM sodium ascorbate in 100% MeOH. Both solutions were mixed 1:1, tightly sealed and incubated at 28 °C at 5 rpm over night.

2.9.2. Coupling to aminoreactive microtiter plates

Deprotection of *t*Boc protected saccharides **5d**–**9d** was reached in 1 M HCl over night at 4 °C. Deprotection was controlled by HPLC (15% acetonitrile, 0.1% formic acid) at 205 nm. MTO-Dowex® M43 anion exchange resin (Supelco, Bellefont, PA, USA) was used to neutralize to pH 7. 1 mM or 10 mM of saccharides **d** were dissolved in 50 μL carbonate buffer (50 mM, pH 9.6) and incubated over night in aminoreactive microtiter plate.

2.9.3. Lectin assay

The saccharide-equipped microtiter plates were washed with PBS and used for lectin binding experiments. ELLA was performed as described previously [13]. Lectin detection followed with 50 μL galactose specific His_6-tagged recombinant His_6CGL2 (50 μg/mL) from *Coprinus cinereus*. ELLA was completed by peroxidase-coupled antibody-detection of the His_6-tag and the following colorimetric measurement. Binding data were analysed by Microsoft Excel® calculations.

3. Results and discussion

Glycosides of GlcNAc carrying either the azide or alkyne functionality as well as two amino-functionalized GlcNAc glycosides were chemically synthesized. Kinetic characterization of β4GalT-1 should reveal the best substrates for preparative-scale enzymatic LacNAc synthesis. Defined azide-, alkyne- and amino-functionalized LacNAc structures were prepared efficiently in mg-scale. Enzymatic LacNAc oligomers synthesis was performed in one-pot with subsequent product isolation by preparative HPLC to obtain defined oligosaccharide chains. In this way, a flexible and well characterized toolbox for the functionalization of surfaces with LacNAc oligomers is obtained employing different immobilization strategies on commercial microtiter plates. According to our previous studies binding efficiencies of azide- and amino-functionalized LacNAc oligomers were tested with the galectin His_6CGL2 [13].

3.1. Chemical synthesis of functionalized β-N-acetylglucosaminides

The chemical synthesis of glycosides was performed in three steps (Scheme 1). In the first step (i) GlcNAc was converted to intermediate **1** according to Horton [23]. Subsequent conversion of **1** (ii) to **2a** was performed according to Ibatullin [24] and (iii) to **2b** and **c** via a Koenigs–Knorr [25–27] glycosidation. In a selective deprotection (iv) Zemplén [28] glycosides **3a**–**d** were obtained. Compound **3e** was prepared according to Sauerzapfe et al. [13]. The saccharide-coupler **4** was prepared according to Anders et al. [29] All compounds were characterized by ^1H, ^{13}C NMR, and, where indicated, IR spectroscopy (see supporting information).

3.2. Kinetic data

In our previous studies we demonstrated that recombinant human β4GalT-1 preferentially accepts hydrophobic aglyca and can be efficiently applied for the synthesis of LacNAc oligomers [13,15,19]. We here further extend the acceptor substrate spectrum for recombinant human β4GalT-1 by six differently functionalized GlcNAc glycosides with hydrophilic and hydrophobic aglyca (Table 1). All GlcNAc glycosides were tolerated by β4GalT-1, but with different kinetics due to their aglycone structure and polarity (see Figs. S1 and S2, supporting information). The comparison of compounds **3a**–**3c** reveals a significant higher affinity as well as

Table 1
Kinetic constants of β4GalT-1 for different C-1 functionalized β-*N*-acetylglucosaminides.

	N-acetyl-β-D-glucosaminide	$K_{m\ app}$ [mM]	$V_{max\ app}$ [U/mg]	$K_{iS\ app}$ [mM]
3a	β-D-GlcNAc-N_3	1.3	0.4	5.7
3b	β-D-GlcNAc-O$(CH_2)_2$-N_3	0.5	0.5	0.04
3c	β-D-GlcNAc-O$(CH_2)_6$-N_3	0.1	0.6	0.003
3d	β-D-GlcNAc-O$(CH_2)_2$-NH-tBoc	0.5	0.8	2.5
3e	β-D-GlcNAc-OCH_2-C_2H	0.9	0.6	5.8
4	β-D-GlcNAc-coupler	0.6	0.3	0.02

slightly higher activity of β4GalT-1 with increasing linker length (Table 1). However, this is accompanied by significant decrease of K_{iS} values indicating a strong substrate inhibition with increasing hydrophobicity. Acceptor substrate **3c** was found to have a 13-fold higher affinity (K_m) compared to the polar acceptor **3a**, concomitant with a severe substrate inhibition (K_{iS}) at a concentration of 0.003 mM. The 33-fold lower K_{iS} value compared to K_m suggests that quantitative conversion of **3c** leading to the corresponding LacNAc derivative may be difficult to achieve.

For comparison, compound **3b** with a medium azide-linker length exhibited a 5-fold increased K_m value compared to **3c** and an order of magnitude lower substrate inhibition, ranging between those for **3a** and **3c**. These results illustrate clearly the impact of linker hydrophobicity on the kinetics of β4GalT-1 and further confirms previous studies on the kinetics for hydrophobic or PEGylated GlcNAc substrates of His_6propeptideβ4GalT-1 [19,30] and of β4GalT from bovine milk [18]. We recently demonstrated that triazole-linked GlcNAc dimers are accepted by the β4GalT-1 (Y284L) mutant for the synthesis of LacDiNAc dimers [31]. In the present study substrate **4** bearing a triazole ring bound to a bulky substituent was accepted with reasonable kinetic constants.

However, with a 30-fold higher substrate inhibition (K_{iS}) compared to K_m substrate **4** is comparable to compound **3c** and may also be difficult to convert quantitatively in synthetic reactions. In contrast, substrate inhibition of β4GalT-1 for the compounds **3d** and **3e** was significantly reduced due the short linkers carrying a *t*Boc-protected amino or an alkyne group. The kinetic characterization of β4GalT-1 for the donor substrate UDP-Gal in the presence of **3a**–**e** and **4**, respectively, showed Michaelis–Menten-type kinetics (data not shown) and gave, apart from **3e**, similar values for K_m and V_{max} (see Table S1, supporting information).

In conclusion, based on detailed comprehensive kinetic studies of human β4GalT-1 with azide- and amino-functionalized acceptor structures for optimization of the enzymatic synthesis of LacNAc derivatives we clearly demonstrate that the activity of β4GalT-1 is influenced by the aglycon-length and hydrophobicity and conditions for synthetic reactions have to be carefully chosen.

3.3. Enzymatic synthesis of LacNAc

The acceptor substrates **3a**–**3e** were further used for the enzymatic synthesis of the corresponding LacNAc products **5a**–**5e** (Scheme 2). Conversion of compound **4** was not further followed since **4** can be easily obtained by "clicking" the corresponding alkyne-terminated linker to the glycoside azide **5a**. The supply of the donor substrate UDP-Gal started from UDP-Glc where indicated and was catalyzed by UDP-glucose 4′-epimerase from *C. jejuni* [21].

The conversion of **3b** and **3c** was strongly impaired by substrate inhibition (Table 1). At a substrate concentration of 5 mM the reaction rate decreased with stronger substrate inhibition constants (Fig. S3, supporting information). Consequently, the reaction conditions were optimized for the conversion of **3c** in particular by a fed-batch technique feeding small amounts of **3c** at distinct time points of conversion.

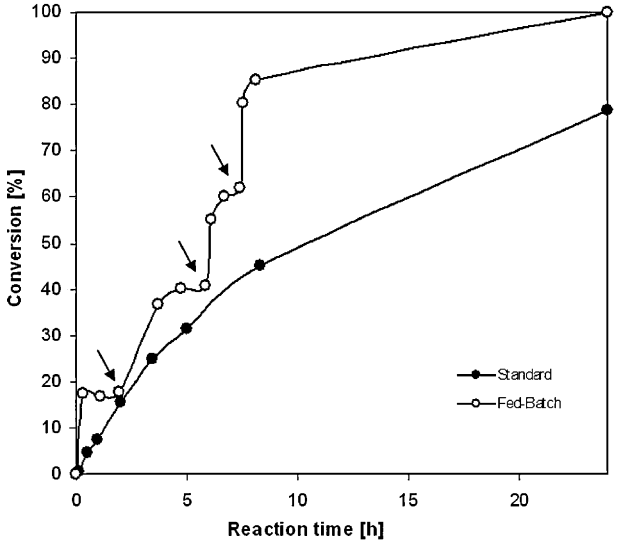


Fig. 1. Conversion of **3c** under standard conditions starting with a substrate concentration of 5 mM (filled circles) and fed-batch conditions (open circles) feeding three portions (arrows) of **3c** (1.25 mM each, with starting concentration of 1.25 mM).

Fig. 1 shows the progress of synthesis under standard conditions (5 mM of **3c** as starting concentration) and the fed-batch synthesis with 1.25 mM **3c** starting concentration and feeding three portions of **3c** (1.25 mM each). Quantitative conversion of **3c** in the same reaction time could only be reached by fed-batch conditions. Reaction conditions were thus optimized for quantitative conversion of **3a**–**3e**. Finally, the products were purified applying semi-preparative HPLC. The total amount of obtained solid products was 300 mg for **5c** and **5d**, respectively, and up to 30 mg for **5a**, **5b**, and **5e**. All LacNAc products were analysed by mass spectrometry and confirmed by NMR spectroscopy (see supporting information).

Product **5b** was previously synthesized in multi-g scale using the bacterial β4Gal-T LgtB from *Neisseria meningitidis* [32] demonstrating the strength of enzymatic glycoconjugate synthesis. In comparison, we here utilized four novel acceptor substrates for the synthesis of LacNAc derivatives by β4Gal-T with optimized synthesis conditions based on substrate kinetics.

3.4. Sequential and one-pot enzymatic synthesis of LacNAc oligomers

For the synthesis of LacNAc oligomers we chose **5c** and **5d** because of their different linker chemistry and good synthesis yields. The sequential synthesis was performed as depicted in Scheme 3. The transfer of GlcNAc onto Gal-terminated oligomers by β3GlcNAcT from *H. pylori* was followed by the reaction of β4GalT-1 [13]. The donor substrate UPD-Gal was generated from UDP-Glc by UDP-glucose 4′-epimerase [21]. The progress of each enzymatic elongation step was monitored by HPLC for complete conversion of the corresponding acceptor substrates. Sequential synthesis yielded LacNAc oligomers with up to three LacNAc units (**9c** and **9d**). The products were characterized by NMR (up to tetrasaccharide **7c** and **7d**) and MS analysis (see supporting information).

The one-pot synthesis of LacNAc oligomers with subsequent product isolation by semi-preparative HPLC was recently developed in our group [15]. The combination of three enzymes proved to be the more economic way due to shorter reaction time and lower enzyme consumption. Scheme 3 shows the potential of one-pot syntheses starting from **5c** or **5d**. The product composition of LacNAc oligomers in a one-pot synthesis with **5c** as starting substrates revealed tri-, penta-, and heptasaccharides as main

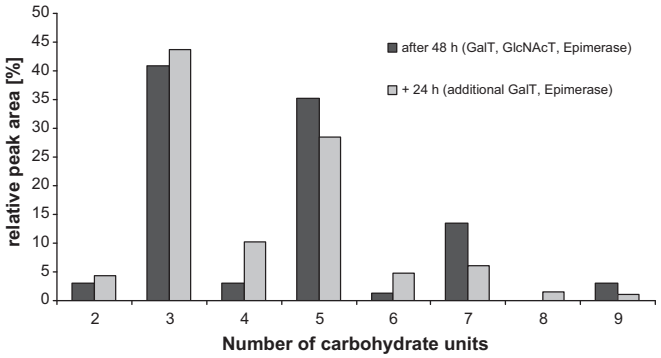


Fig. 2. Product distribution of a one-pot synthesis of LacNAc oligomers with **5c** as starting substrate.

products (Fig. 2). Gal-terminated structures (even numbered) were the minor fractions, although β4GalT-1 was present in excess. The outcome of this experiment reflects the influence of the linker type and the chain lengths on the course of reaction. The occurrence of uneven-numbered oligomers as main products suggests that β3GlcNAc-T is highly efficient for the conversion of the corresponding acceptor substrates.

In contrast, substrate conversion by β4GalT-1 seems to be limited which may be due to the unfavourable influence of the hydrophobic linker on enzyme kinetics (Table 1). As discussed above, compound **3c** shows a strong substrate inhibition which can be overcome by a fed batch synthesis of **5c** (Fig. 1). However, in one-pot synthesis GlcNAc-terminated products (tri-, penta-, and heptasaccharide) cannot be efficiently converted by β4GalT-1. Even subsequent incubation of the product mixture with β4GalT-1 increased the amount of the even-numbered fractions only slightly (Fig. 2). In contrast, synthesis starting from **5d** gave a product mixture with a higher amount of Gal-terminated oligomers (see Fig. S4 in supporting information) due to the more favourable substrate kinetics. Isolation of single LacNAc oligomers from the one-pot product mixtures was accomplished by semi-preparative HPLC (see Figs. S5 and S6, supporting information). Single fractions of defined oligosaccharides were obtained and confirmed by MS and NMR.

3.5. Immobilization of LacNAc oligomers and lectin binding experiments

To explore linker and concentration dependent lectin-binding characteristics the products **5d**–**9d** carrying *t*Boc-linker group were deprotected as previously described [13] and subsequently immobilized onto the commercial available amino-reactive microtiter plate (Scheme 1, supporting information).

Binding of the galactose specific fungal His_6CGL2 lectin was only observed with 10 mM immobilization concentration of **5d**–**9d** (Fig. 3) in the Enzyme-Linked-Lectin-Assay (ELLA). However, lectin binding was strongly dependent on the length of the saccharides as a significant signal was only obtained for **8d** and **9d**. We concluded that the linker length was not suitable for the surface display of shorter (<pentasaccharide) carbohydrate chains in a microtiter plate. A clear dependence of lectin binding from the linker length and the immobilization concentration was reported for a glycan micro-array on a glass surface [9]. The application of an additional spacer molecule could improve the display as reported previously [33,34]. However, incorporation of an additional linker is too complex for the immobilization of amino-terminated saccharides onto an amino-reactive microtiter plate.

Instead, the saccharides **5c**–**9c** carrying a C_6-azide linker were utilized for immobilization by click chemistry [33,35]. The

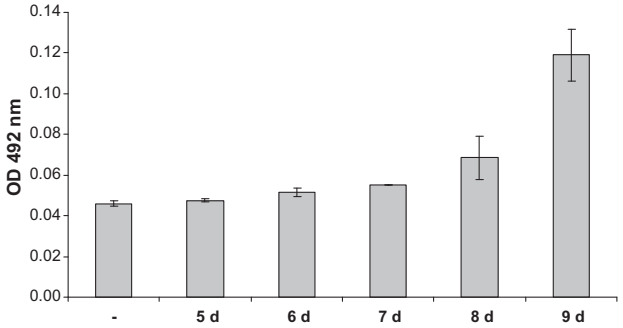


Fig. 3. His_6CGL2 binding to saccharides **5d**–**9d** at 10 mM immobilization concentration.

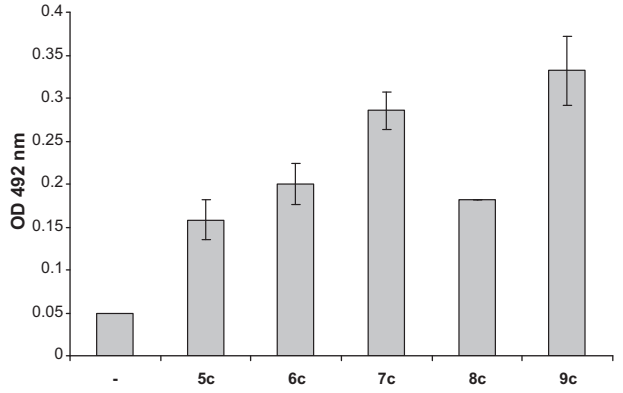


Fig. 4. His_6CGL2 binding on clicked saccharides **5c**–**9c** (1 mM of each LacNAc oligomer).

functionalization of the amino-reactive microtiter plate with propargylamine resulted in an alkyne-functionalized surface ready for coupling **5c**–**9c** (see Scheme S2, supporting information). The click chemistry led to colorimetric signals with low background sufficient to evaluate the influence of carbohydrate chain length onto binding of the fungal lectin His_6CGL2 for each product (Fig. 4).

With increasing chain length higher binding signals for products **5c**–**7c** and **9c** were observed. For galactose terminated di-LacNAc **7c** and tri-LacNAc **9c** binding of His_6CGL2 was significantly improved compared to LacNAc **5c**. Odd numbered oligosaccharides **6c** and **8c** gave comparably lower signals due to weaker affinity of His_6CGL2 to GlcNAc-terminated structures [13,36,37]. However, the higher signal of **6c** (trisaccharide with terminal GlcNAc) compared to **5c** (LacNAc) was not expected for His_6CGL2 binding according to our previous results [13] and may be attributed to different linker chemistry. Similar linker effects on lectin binding can be followed in the lectin data bank of the Consortium of Functional Glycomics [http://functionalglycomics.org].

An optimized coupling strategy has also to be considered for the immobilization of biomolecules onto hydrogels. Hydrogels, made of poly[(ethylene oxide)-*stat*-(propylene oxide)] (sP(EO-*stat*-PO), avoid non-specific protein adsorption and are therefore excellent materials for surface modifications of biomaterial surfaces with biomolecules [38–41].

4. Conclusion

A combinatorial chemo-enzymatic synthesis of LacNAc oligomers for subsequent lectin binding is presented. Functionalized GlcNAc building blocks were selected for the synthesis of LacNAc based on their kinetic characteristics as acceptor substrates of recombinant human β4GalT-1. Following a successive

and a one-pot synthesis strategy combinatorial biocatalysis with three enzymes was applied to obtain functionalized LacNAc oligomers. Immobilization of LacNAc oligomers onto aminoreactive surfaces via 1,3 dipolar cycloaddition or an amino-linker revealed linker length as well as repeated LacNAc-units to promote galectin binding. This innovative and flexible toolbox system of carbohydrate ligands can be used for the biofunctionalization of hydrogel-coated biomaterial surfaces and is therefore a promising step towards biomedical applications.

Acknowledgements

Financial support by the DFG (EL 135/8-1, MO 682/8-1) and by the excellence initiative of the German Federal and State Governments through ERS@RWTH Aachen University is gratefully acknowledged. We thank Dr. Wolfgang Bettray (Institute of Organic Chemistry, RWTH Aachen University) for ESI-MS measurements and Janine Schmitz for the syntheses of saccharide **5c**.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2012.02.002.

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