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Subsequent Enzymatic Galactosylation and Sialylation Towards Sialylated Thomsen–Friedenreich Antigen Components

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Abstract: Sialyloligosaccharides of the type Neu5Acα2-3Galβ1-3GalNAc and a range of corresponding motifs play an important role in nature and are found in gangliosides, Lewis type I structures and the sialyl-Thomsen–Friedenreich antigen occurring in higher animals, viruses, bacteria, protozoa and pathogenic fungi. There is considerable interest to evaluate the significant functionalities of these glycostructures, and here we present a chemoenzymatic approach by a facile synthesis of such motifs. Employing chemoenzymatic methods, several modified Galβ1-3GalNAc derivatives were synthesized. Modifications were introduced at the stage of the monomeric building blocks prior to formation of the disaccharides by means of four different β-galactosidases from bovine testes, *Bacillus circulans* and *Xanthomonas manihotis* as well as the phosphorylase from *Bifidobacterium bifidum*. Finally, the modified disaccharide derivatives could be efficiently sialylated using the recombinant trans-sialidase from *Trypanosoma cruzi*.

Keywords: carbohydrates; enzyme catalysis; glycosylation; regioselectivity; sialic acid

Introduction

More than 40 different sialic acid derivatives occur in nature on carbohydrate residues, often masking the non-reducing terminus of the oligosaccharide chains. Thus they participate in recognition processes,[1] and it is well known that pathogens such as bacteria and viruses like the influenza virus are able to recognize and bind selectively sialic acids on cell surfaces.[2] The Thomsen–Friedenreich antigen (T-antigen) Galβ1-3GalNAc is the central component of the core A structure in *O*-glycoproteins and ubiquitously present in organisms. It does not only present the preliminary stage of complex oligosaccharides of the mucin type but also appears unmasked or preliminarily sialylated on the surface of cancer cells. For this reason compounds with such motifs are important in cancer research as potential leads *en route* to vaccines or antimetastatically active cancer drugs.[3] The T-antigen determinant is a part of ganglio-glycosphingolipides, and the sialylated derivative Neu5Acα2-3Galβ1-3GalNAc (sialyl-T-antigen determinant) is present on the non-reducing end of the gangliosides G_{Q1b}, G_{D1a} and G_{T1b}.

Since the first observation of myelin-associated glycoprotein (MAG), its key function for the formation and maintenance of the myelin membrane was proposed.[4] In the growth of neurites MAG has a bifunctional effect. On the one hand, neurite outgrowth of neurons in newborns will be promoted, on the other hand, it strongly inhibits neurite outgrowth of neurons in the adult species.[5] In this way the adult nervous system is protected from wild branching of neurons. However, the drawback is that, after injury, the central nervous system cannot be repaired. Substrates binding strongly to MAG prevent their interaction with the native receptor and in this way prevent MAG's inhibition of neurite outgrowth. In conjunction with respective growth factors the recovery of injured nerve tissue might be achievable in this way. MAG's change of function originates from a different constitution of gangliosides: in the neonatalic nervous system the trisaccharide G_{D3} prevails without having the Neu5αAc2-3Galβ1-3GalNAc motif (Figure 1) but

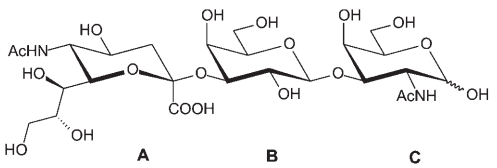


Figure 1.

with the beginning of myelination the hexasaccharide G_{D1a} containing that motif takes the lead.

In vitro MAG prefers binding to glycoconjugates containing the Neu5Ac2-3βGal1-3GalNAc motif[6,7] for example, the gangliosides $G_{Q1b,}$ G_{D1a} and G_{T1b} found in membranes of nervous cells.[8] Any modifications in the *N*-acetylneuraminic acid moiety will prevent recognition by MAG, but for a systematic recognition a facile synthesis of the above mentioned motifs is required (Figure 1). So far the octasaccharide ganglioside $G_{Q1b\alpha}$ is the most potent antagonist of MAG identified.[9]

The parasitic trans-sialidase from *Trypanosoma cruzi*, the pathogen responsible for Chagas' disease, catalyzes an unusually efficient sialyltransfer, transferring terminal Neu5Ac units from sialo-glycoconjugates to sialylate its own surface mucins and thus mask its own epitopes.[10–13] The exact catalytic mechanism of trans-sialylation is presently under study.[14–16] Correct binding of sialic acid donors to trans-sialidase (TS) may trigger a conformational change in the enzyme that induces the conditions for formation of a ternary complex. Acceptor binding would displace the water molecules from the TS catalytic cleft before formation of the oxocarbonium ion or sialyl-enzyme intermediate takes place, facilitating the selective transfer reaction. By using NMR spectroscopy it was demonstrated that inactive *T. cruzi* TS has a carbohydrate recognition domain for β-Gal residue that is formed only after a conformational switch triggered by prior sialoside binding.[17]

In this work, we present chemoenzymatic methods for the synthesis of the sialyl T-antigen determinant Neu5Acα2-3Galβ1-3GalNAc and derivates thereof. Employing β1-3-galactosidases from various sources or a phosphorylase, the building block C, being GalNAc or a modification of it, was glycosylated with building block B. The disaccharides obtained were sialylated in the final step utilizing trans-sialidase of *T. cruzi*. Previously sialylated T-antigen determinant structures were efficiently synthesized by Kunz et al.[18,19] as well as by Schmidt et al.[20] Our enzymatic reaction sequence presented here is straightforward, fast and efficient, affording the desired products in absolute regio- and stereoselectivity.

Results and Discussion

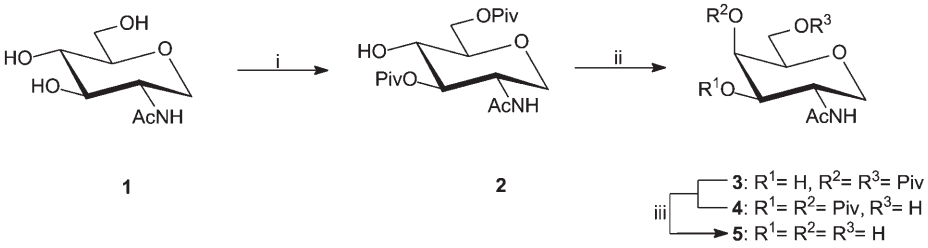
Synthesis of Monosaccharide Acceptors

Radical dehalogenation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[21] according to Bramford et al. followed by deprotection yielded 2-acetamido-1,5-anhydro-glucitol **1**.[22] Regioselective acylation furnished the 3,6-dipivaloate **2**, the structure of which was confirmed by HMBC NMR. Synthesis of the corresponding triflate succeed quantitatively in situ in dichloromethane/pyridine (20:1), followed by intramolecular attack and migration of the pivaloyl groups under inversion at position 4.[23] The *galacto*-configured 4,6- (**3**) and 3,4-dipivaloates **4** in a ratio of 9:1 were deprotected and yielded the 2-acetamido-1,5-anhydro-galactitol **5** in a total yield of 48% over six steps based on the glycosyl chloride (Scheme 1).

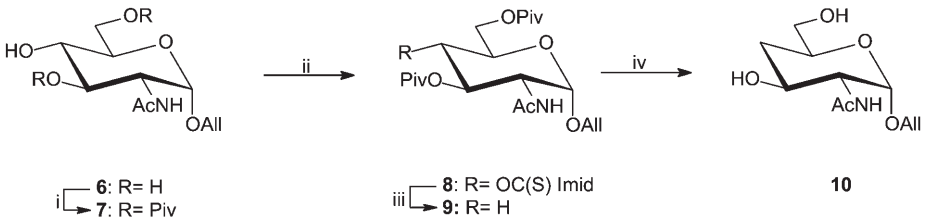
Starting with allyl 2-acetamido-α-D-glucopyranoside **6**[24] pivaloylation in positions 3 and 6 could be achieved to give crystalline **7** in 77% yield with only 6% of the tripivaloate as side product. Substitution of a triflyl group by $NaBH_4$ allows the deoxygenation of primary and secondary positions.[25] Thus the triflyl group was introduced quantitatively at position 4 of compound **7**, and subsequent deoxygenation with $NaBH_4$ in acetonitrile gave compound **9** in only 48% yield. Consequently, the Barton–McCombie deoxygenation of thiocarbonyl compounds with Bu_3SnH was selected.[26] Employing tetrahydrofuran as solvent the acylation of **7** with 1,1′-thiocarbonyldiimidazole (TCDI)[27] yielded compound **8** in very good yield (97%), and the radical reduction with tributyltin hydride without a radical starter furnished the 4-deoxy compound **9** in high yields. The subsequent deprotection of the pivaloyl groups to give **10** was unusually slow and required considerable amounts of catalyst (Scheme 2).

Synthesis of Disaccharides

The modified *N*-acetyl-galactosamines needed to be galactosylated chemoenzymatically in order to obtain compounds corresponding to the T-antigen structure



Scheme 1.



Scheme 2.

Galβ1-3GalNAc. Thus, modified monosaccharide acceptors were glycosylated using *p*-nitrophenyl β-D-galactopyranoside (*p*NP-Gal) and α-D-galactopyranosyl phosphate (Gal-P) donors, respectively. The glycosidic linkages were established by means of β-galactosidases from bovine testes, *Bacillus circulans* (bgac-gen) and *Xanthomonas manihotis,* as well as by the phosphorylase from *Bifidobacterium bifidum.*

György et al. carried out first synthetic investigations employing a cell-free extract from bovine testes. *N*-Acetyl-D-glucosamine was galactosylated using lactose as donor at positions 3 and 4 in a ratio of 10:1 in a yield of 2.3%.[28] Since the β-galactosidase is quite expensive, an extract from bovine testes was isolated according to a modified procedure by Distler and Jourdian.[29] This crude extract contained a β-hexosaminidase as impurity, thus any β-glycosidic linkage in the acceptor structures such as *N*-acetyl-D-glucosamine or galactosamine would be hydrolyzed, preventing their galactosylation.[30]

The Gram-negative bacteria *Xanthomonas manihotis* contains eight *exo*-glycosidases, including a β-galactosidase which preferably cleaves the β1-3 linkage.[31,32] Whereas the acceptor GlcNAc was galactosylated only in position 3 in a yield of 22%, GalNAc furnished both the β1-3 and β1-6 regioisomers in a ratio of 7:3 in a similar yield of 25%.[33,34] The enzyme displays an activity maximum at a pH value between 5.0 and 6.5. The addition of DMSO, DMF or acetone was not tolerated, whereas a content of 10% acetonitrile or 20% *tert*-butanol had no noteworthy effect on the activity of the enzyme. The best temperature for the incubation proved to be 37 °C, and a positive effect was observed by addition of 0.05‰ of bovine serum albumin (BSA). The optimal conditions employed in this study were sodium acetate buffer (100 mM, pH 5.5, 0.05‰ BSA) with 10% acetonitrile and a temperature of 37 °C.

In addition to the two well known and commercially available galactosidases I and II, Ito and Sasaki observed in *Bacillus circulans* (mutant ATCC31382) a novel β-galactosidase. They successfully cloned the responsible bgaC-gen and expressed it in *Escherichia coli.*[35] In contrast to the commercially available β-galactosidase, the new β-galactosidase favourably hydrolyzed a β1-3-linked galactose and established this linkage regioselectively under transglycosylation conditions as well.[36–38] Optimal reaction conditions proved to be 20% DMF in potassium phosphate buffer (100 mM, pH 6.0) at an incubation temperature of 50 °C. During incubations of relatively sensitive GalNAc derivatives decomposition products were observed, thus reactions were conducted at 37 °C without considerably slowing down the reaction.

Bifidobacterium bifidum located in the human intestinal tract encloses several enzymes digesting mucins. From the mutant *B. bifidum* DSM 20082 the non-Leloir glycosyl transferase β1-3-galactosyl-*N*-acetylhexosamine phosphorylase was isolated which specifically cleaves a galactose residue from position 3 of GlcNAc and GalNAc to give galactose 1-phosphate and the corresponding *N*-acetyl-hexosamine. So far, this is the only known phosphorylase recognizing a terminal galactose moiety.[39] In the reverse reaction *N*-acetyl-D-galactosamine and glucosamine could be galactosylated with galactose 1-phosphate at position 3.[40] The enzyme used was a cell-free crude extract containing a β-hexosaminidase. Due to the small amount of the enzyme available, no attempts were undertaken to optimize the reaction conditions. Therefore previously elaborated conditions with 1.2 equivalents of the acceptor in imidazole buffer (25 mM) at pH 6.8, 37 °C and 20 h were employed.

The reaction conditions optimized during hydrolyses were employed with allyl 2-acetamido-α-D-galactopyranoside (α-Allyl-GalNAc) as a sample acceptor to examine the conditions for transglycosylation. In all hydrolase reactions an excess of donor led to some extent of auto-galactosylation of the donor *p*NP-Gal and galactosylation of the hydrolysis product D-galactose. These side reactions were not observed using an excess of acceptor.

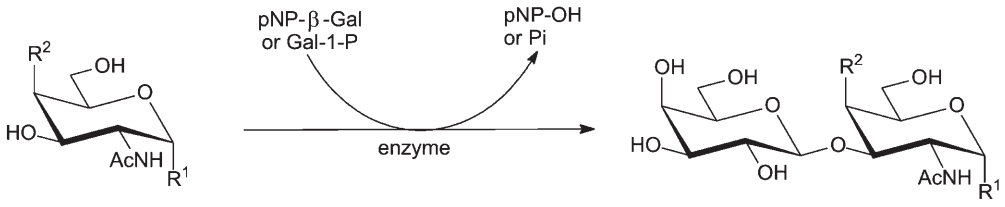
Reactions catalyzed by β-galactosidase from bovine testes and *X. manihotis* showed a maximum of product formation after 48–55 h incubation time, whereas the optimum yield using β-galactosidase from *B. circulans* was reached after 4.5 h. The reactions were stopped by heating to 90 °C, lyophilization of the solvent followed by purification using size exclusion chromatography.

The galactosylation of allyl-GalNAc employing the β-galactosidases from *X. manihotis* and *B. circulans* did not only give the desired β1-3-disaccharides, but a further major product was observed. The hydrolyzed

donor and excess acceptor were removed by size exclusion chromatography on Biogel P2 and the disaccharide fraction subsequently acetylated under standard conditions. Purification on silica gel yielded both the β1-3 product and the β1-6 derivative in yields of 33% and 22%, respectively.

The chemoenzymatic galactosylation of the allyl (**11**) and methyl α-glycopyranosides (**12**) of GalNAc in the presence of galactosidase from bovine testes gave the β1-3-linked products. The yields for the galactosylation could be increased from 22%[41] to a convincing 67%, proving this method to be superior to previously published methods in efficiency and overall yield. The regioselective galactosylation of **12** was successfully performed in a yield of 66% (Scheme 3).

The chemoenzymatic galactosylation of **11** and **12** with galactose 1-phosphate as donor in the presence



11-14 **15-18**

	R^1	R^2	Yield
11	OAll	OH	**15**
Bovine testes			67 %
Xanthomonas manihotis			21 %
Bacillus circulans			34 %
Bifidobacterium bifidum			43 %
12	OMe	OH	**16**
Bovine testes			66 %
Xanthomonas manihotis			24 %
Bacillus circulans			33 %
Bifidobacterium bifidum			44 %
13	H	OH	**17**
Bovine testes			70 %
Xanthomonas manihotis			19 %
Bacillus circulans			31 %
Bifidobacterium bifidum			41 %
14	OAll	H	**18**
Bovine testes			67 %
Xanthomonas manihotis			22 %
Bacillus circulans			37 %
Bifidobacterium bifidum			41 %

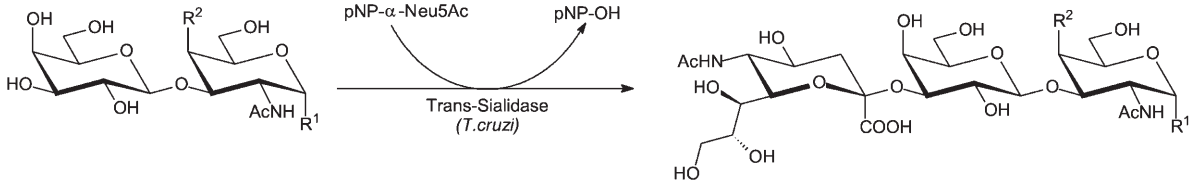
Scheme 3. Transglycosylations with β1-3-galactosidases from bovine testes, *Xanthomonas manihotis, Bacillus circulans* and the phosphorylase from *Bifidobacterium bifidum.*

of phosphorylase from *Bifidobacterium bifidum*, yielded solely the β1-3-linked products in yields of 43% and 44%, respectively. The yields obtained were lower than when using galactosidase from bovine testes. It may be assumed that steric effects in the active center between the substrate and enzyme could be responsible. Using β-galactosidase from *Xanthomonas manihotis* and *Bacillus circulans*, the yields of the desired β1-3-linked products were comparable. In the presence of *X. manihotis* the β1-6-linked byproduct was observed in small amounts, whereas in the presence of *B. circulans* a considerable amount of the same byproduct was found. A comparable regioselectivity was observed by Ajisaka et al. employing *B. circulans* with D-GalNAc and D-Gal-*O*-Me acceptors.[42] For facile purification and isolation the β1-6 byproduct could be hydrolyzed by β-galactosidase from *Escherichia coli* without affecting the β1-3 products **15** and **16**, respectively.

It was expected that galactitol **13** missing an aglycone structure would provide insight into the role of the aglycone concerning yield and regioselectivity. Apparently, the aglycone did not show any effect or consequence for the binding of the acceptor in the enzyme pocket. The galactosylation employing the enzymes from bovine testes or *Bifidobacterium bifidum* did not show any response to the missing aglycone structure regarding yield or regioselectivity in comparison to **11** and **12**. Using galactosidase from *Xanthomonas manihotis* the yield was comparable to the other acceptors investigated, however, the formation of a by-product was observed in higher amounts. Possibly stabilizing steric or hydrophobic interactions of the aglycone residue in the binding pocket of the enzyme are absent in **13**, which might favour the orientation of the acceptor towards a galactosylation at position 3. The chemoenzymatic galactosylation of the 4-deoxy derivative **14** occurred exclusively at position 3 in yields comparable to the glycosylation of **11** or **12.** In general, the β-galactosidases from *X. manihotis* and *B. circulans* glycosylated the primary position of *galacto*-configured acceptors, however not those of the respective *gluco*-configured components. In conclusion, the equatorial orientation of the hydoxy group in position 4 of GlcNAc was proven to be not responsible for the regioselective galactosylation at position 3. In contrast, apparently the axial hydroxy group of GalNAc derivatives seems to disturb the recognition by the enzyme leading to a mixture of regioisomers.

Synthesis of Sialylated Oligosaccharides

Employing *p*NP-*N*-acetyl-neuraminic acid, the disaccharide derivatives obtained (Scheme 3) were used as acceptors in enzymatic reactions catalyzed by the trans-sialidase derived from *T. cruzi*. It is assumed that the enzyme has two subsites in the active center: the sialic acid binding site and the galactose binding site, the latter being absent in bacterial and viral sialidases. Binding of the sialic acid moiety of the donor may trigger a conformational switch, generating the β-galactosyl acceptor binding site.[43] The transfer activity depends strongly on incubation conditions, such as pH, temperature and time. Trans-glycosylation with *T. cruzi* took place with equimolar amounts of substrate and in eight hours incubation time as developed previously.[44] Higher enzyme activities were achieved

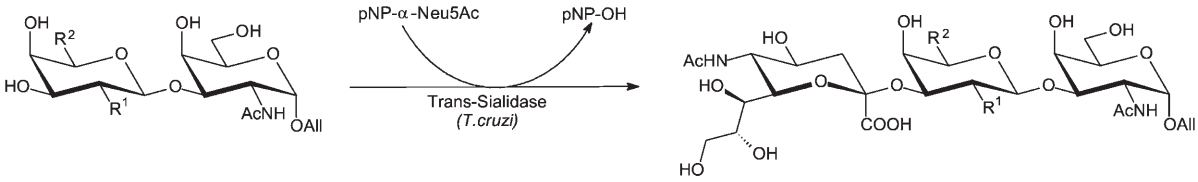


15-18 **19-22**

Gal β1-3GalNAc structures	R^1	R^2	Sia-Gal-GalNAc* derivatives	Yield
15	OAll	OH	**19**	46%
16	OMe	OH	**20**	30%
17	H	OH	**21**	44%
18	OAll	H	**22**	43%

GalNAc* = modified GalNAc.

Scheme 4. Sialylation of GalNAc-modified disaccharides.



23-25 26-28

Gal*-GalNAc structures	R^1	R^2	Sia-Gal*-GalNAc derivatives	Yield
23	OH	H	**26**	46%
24	OH	CH_3	**27**	30%
25	H	CH_2OH	**28**	43%

Gal* = modified Gal.

Scheme 5. Trans-sialylation with trans-sialidase of Galβ1-3GalNAc derivatives modified in the terminal Gal residue.

in a buffer system of Tris-HCl buffer at pH 7.5 and a temperature of 23 °C.

Sialylation of GalNAc-modified disaccharides employing trans-sialidase led to modified determinants shown in Scheme 4 and Scheme 5 in fairly good yields of 30 to 46 %. In reactions catalyzed by trans-sialidase of *T. cruzi* a galactose linked at position 1 is a prerequisite. No significant differences were observed in the synthesis of trisaccharides **19**, **21** and **22**, showing no influence of the aglycone structure in the recognition by trans-sialidase of *T. cruzi*. In contrast, the yield of trisaccharide **20**, with a methyl residue as aglycone, turned out to be somewhat lower.

Previous work reported enzymatic sialylations with trans-sialidase (*T. cruzi*.), employing a Gal residue in the acceptor site.[44] In other studies reactions with the immobilized enzyme were investigated.[45] In our further work we could use 2-deoxy- (**25**) and 6-deoxy derivatives (**24**) of D-galactose as well as L-arabinose (**23**) as building block B[46] to investigate the influence of the hydroxy groups in positions 2 and 6 or a missing CH_2OH group on the transfer employing trans-sialidase.

Apparently, as deduced from the respectable yields of the trisaccharide **28** modified in position 2 of building block B, it can be assumed that the lack of the hydroxy group in this position does not influence the transfer. Further, use of L-arabinose **23** and 2-deoxy derivative **25** as building blocks B again does not change the sialylation to give average yields of 46 % for **26** and 43 % for **28**.

In contrast, the terminal fucose residue, being less polar than arabinose and the 2-deoxy-galactose, can be considered to show a lower binding affinity to the active center of the enzyme, and thus the yield of **27** is somewhat lower than that of **26** or **28**.

Conclusions

A series of modified *N*-acetyl-galactosamine acceptors has been prepared and galactosylated employing three β-galactosidases from bovine testes, *Bacillus circulans*, *Xanthomonas manihotis* as well as the phosphorylase from *Bifidobacterium bifidum*. Their subsequent sialylation was performed with trans-sialidase (*T. cruzi*) in good yields to give building block C-modified sialylated Thomsen–Friedenreich antigen components. Furthermore, Galβ1-3GalNAc structures modified in the terminal building block were convincingly sialylated to give the corresponding Sia-T antigen with modified B building units.

Experimental Section

General Remarks

Commercially available starting materials were used without further purification. Solvents were dried according to standard methods. Purifications of the products were carried out by column chromatography using Merck silica gel 60 (230–400 mesh). The enzymatic reactions were incubated in a thermomixer Comfort (Merck) at 600 rpm. The NMR spectra were recorded on a Bruker AMX-400 (100.62 MHz for ^{13}C) or DRX-500 (125.83 MHz for ^{13}C) spectrometer. All chemical shifts are quoted in ppm downfield from TMS or referred to the characteristic signals of the used solvents $CHCl_3$ in $CDCl_3$ (7.24 ppm), MeOH-d_3 in [D_4]MeOH (3.35 ppm) or HDO in D_2O (4.63 ppm). Mass spectra were recorded on a Bruker MALDI-Tof Biflex III.

Trans-sialylation with Recombinant Trans-sialidase from *Trypanosoma cruzi* (Method 1)

A solution of 10 mg *p*NP-Neu5Ac and 10 mg acceptor in 1.0 mL degassed incubation buffer (100 mM Tris/HCl, pH 7.5, 50 μg BSA, 0.02% NaN_3) was incubated with 100 μL recombinant TS (5.7 mg/1 mL) at 23 °C for 8 h. The reaction was monitored by TLC (butanol/acetic acid/water, 5:2:2). After completion of the reaction, the enzyme was denatured and centrifuged before the supernatant was lyophilized. The dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16×900 mm) with water.

Enzymatic Transglycosylation with β-Galactosidase of Bovine Testes (Method 2)

A solution of donor *p*NP-galactopyranoside and acceptor (10 equivalents) in incubation buffer (McIlvaine-buffer 50 mM, pH 4.3) was incubated with β-galactosidase of bovine testes (4.7 U/mmol) at 37 °C for 50 h. After completion of the reaction, the enzyme was denatured by heating to 90 °C. The liberated *p*NP-OH was extracted with ethyl acetate, and the aqueous layer containing the product was lyophilized. The dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16×900 mm). First elution was with an ammonium hydrogen carbonate buffer (25 mmol), and subsequently water was used. Buffer salts were removed using a short mixed bed ion exchange chromatography column.

Enzymatic Transglycosylation with β-Galactosidase of *Xanthomonas manihotis* (Method 3)

A solution of donor *p*NP-galactopyranosid and acceptor (3 equivalents) and small amounts BSA in sodium acetate incubation buffer (100 mM, pH 5.5, 10% acetonitrile) was incubated with β-galactosidase of *Xanthomonas manihotis* (230 U/mmol) at 37 °C for 50 h. After completion of the reaction, the enzyme was denatured by heating to 90 °C. The solution was diluted with the ten-fold volume of sodium phosphate buffer (50 mM, pH 7.0, 1 mM magnesium chloride). To this solution the β-galactosidase of *Escherichia coli* (250 U/mmol) was added and the mixture incubated for 4 h at 37 °C. The pH value was then adjusted to 4.3 using citric acid and the enzyme was denatured. The liberated *p*NP-OH was extracted with ethyl acetate and the aqueous layer containing the product was lyophilized. The dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16×900 mm). First elution was with an ammonium hydrogen carbonate buffer (25 mmol), and subsequently water was used. Buffer salts were removed using a short mixed bed ion exchange chromatography column.

Enzymatic Transglycosylation with β-Galactosidase of *Bacillus circulans* (Method 4)

A solution of donor *p*NP-galactopyranoside and acceptor (3 equivalents) in potassium phosphate incubation buffer (100 mM, pH 6.0, 20% *N,N*-dimethylformamide) was incubated with β-galactosidase of *Bacillus circulans* (bgaC-Gen, 5.9 U/mmol) at 37 °C for 4.5 h. After completion of the reaction, the enzyme was denatured by heating to 90 °C. The solution was diluted with the ten-fold volume of sodium phosphate buffer (50 mM, pH 7.0, 1 mM magnesium chloride). To this solution the β-galactosidase of *Escherichia coli* (250 U/mmol) was added and the mixture incubated for 4 h at 37 °C. The pH value was adjusted to 4.3 using citric acid and the enzyme was denatured. The liberated *p*NP-OH was extracted with ethyl acetate and the aqueous layer containing the product was lyophilized. The dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16×900 mm). First elution was with an ammonium hydrogen carbonate buffer (25 mmol), and subsequently water was used. Buffer salts were removed *via* a short mixed bed ion exchange chromatography column.

Enzymatic Glycosylation with Phosphorylase of *Bifidobacterium bifidum* (Method 5)

A solution of the donor galactose-1-phosphate and acceptor (1.2 equivalents) in imidazole incubation buffer (25 mM, pH 6.8 L) was incubated with phosphorylase of *Bifidobacterium bifidum* DSM 20082 (30 μL mmol^{-1}) at 37 °C for 4 h. After completion of the reaction, the enzyme was denatured by heating to 90 °C. After centrifugation the supernatant containing the product was lyophilized. The dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16×900 mm). First elution was with an ammonium hydrogen carbonate buffer (25 mmol), and subsequently water was used. Buffer salts were removed *via* a short mixed bed ion exchange chromatography column.

^1H, ^{13}C NMR data, MS data and combustion analyses for all compounds are given in the Supporting Information.

2-Acetamido-1,5-anhydro-2-deoxy-3,6-di-*O*-pivaloyl-D-glucitol (2)

A solution of 2-acetamido-1,5-anhydro-2-deoxy-D-glucitol (**1**)[22] (1.60 g, 7.80 mmol) was dissolved in dry dichloromethane (8 mL) and dry pyridine (16 mL). After cooling the solution to −2 °C pivaloyl chloride (2.88 mL, 23.4 mmol, 3 equivalents) was added. The mixture was stirred at 0 °C for 5.5 h. The reaction was stopped with methanol and diluted with dichloromethane. The organic layer was washed with aqueous sodium hydrogen carbonate, aqueous sodium chloride, and the organic layer was dried over $MgSO_4$. After removal of the solvent under reduced pressure the product was purified by column chromatography (silica gel, petroleum ether/ethyl acetate 1:1) to give compound **2** as an amorphous solid; yield: 2.38 g (6.37 mmol, 82%); $[\alpha]_D^{20}$: −24° (*c* 0.6, $CHCl_3$).

2-Acetamido-1,5-anhydro-2-deoxy-4,6-di-*O*-pivaloyl-D-galactitol (3) and 2-Acetamido-1,5-anhydro-2-deoxy-3,4-di-*O*-pivaloyl-D-galactitol (4)

A solution of **2** (2.09 g, 5.60 mmol) in dry dichloromethane (45 mL) and dry pyridine (2.4 mL) was cooled to −35 °C. Trifluoromethanesulfonic anhydride (1.2 mL, 7.1 mmol, 1.3 equivs.) was added dropwise to the solution and warmed up to 0 °C. After stirring for 8 h water (6.0 mL) was added and the solution was refluxed for 42 h. The reaction mixture was diluted with dichloromethane. The organic layer was washed with aqueous sodium hydrogen carbonate and water. The solvent was dried over $MgSO_4$, filtered, and the organic layer was removed at reduced pressure. The product was purified by column chromatography (silica gel, toluene/acetone 5:2) to give **3** and **4** as an amorphous solid; yield: 1.81 g (4.85 mmol, 87 %); ratio **3**:**4** = 9:1 (from ^{1}H NMR).

2-Acetamido-1,5-anhydro-2-deoxy-D-galactitol (5)

To a solution of **3** and **4** (1.68 g, 4.50 mmol) in dry methanol (40 mL) sodium methoxide (40 mg) was added and stirred at room temperature for 30 h. After neutralization with Amberlite IR 120, H^+, the solution was filtered, dried over $MgSO_4$, and the solvent was removed under reduced pressure. Recrystallization was from methanol gave **5** as a colourless solid; yield: 855 mg (4.17 mmol, 93 %): mp 117 °C; $[\alpha]_D^{20}$: +49° (*c* 1, MeOH).

Allyl 2-Acetamido-2-deoxy-3,6-di-*O*-pivaloyl-α-D-glucopyranoside (7) and Allyl 2-Acet-amido-2-deoxy-3,4,6-tri-*O*-pivaloyl-α-D-glucopyranoside (7a)

A solution of allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**6**)[24] (17.5 g, 67.0 mmol) in dry dichloromethane (70 mL) and dry pyridine (140 mL) was cooled to 0 °C. Pivaloyl chloride (24.7 mL, 20 mmol, 3 equivs.) was added to the solution. The reaction was stopped with methanol and diluted with dichloromethane (840 mL). The organic layer was washed with aqueous sodium hydrogen carbonate, water and sodium chloride, dried over $MgSO_4$, filtered and then the solvents removed at reduced pressure. The residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate 2:1) to give the products **7** and **7a**.

Allyl 2-acetamido-2-deoxy-3,6-di-*O*-pivaloyl-α-D-glucopyranoside (7): Yield: 22.2 g (51.5 mmol, 77 %); colourless crystals; $[\alpha]_D^{20}$: +60° (*c* 1, $CHCl_3$); mp 49 °C.

Allyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-pivaloyl-α-D-glucopyranoside (7a): Yield: 2.06 g (4.01 mmol, 6 %); amorphous solid; $C_{26}H_{43}NO_9$: 513.63; $[\alpha]_D^{20}$: +93° (*c* 1, $CHCl_3$).

Allyl 2-Acetamido-2-deoxy-3, 6-di-*O*-pivaloyl-4-*O*-thiocarbonylimidazoyl-α-D-glucopyranoside (8)

A solution of **7** (2.24 g, 5.22 mmol) and 1,1-thiocarbonyldiimidazole (TCDI) (2.20 g, 12.3 mmol, 2.4 equivs.) in tetrahydrofuran (40 mL) was refluxed for 7 h. The solvent was evaporated and the residue was dissolved in dichloromethane. The organic layer was washed with hydrochloric acid (2 M), aqueous sodium hydrogen carbonate and water. The solvent was dried over $MgSO_4$, filtered and then removed under reduced pressure to give **8** as a yellow solid; yield: 2.72 g (5.04 mmol, 97 %); mp 107 °C; $[\alpha]_D^{20}$: +64° (*c* 0.55, $CHCl_3$).

Allyl 2-Acetamido-2,4-dideoxy-3,6-di-*O*-pivaloyl-α-D-*xylo*-hexopyranoside (9)

The reaction was carried out under an argon atmosphere. Tributyltin hydride (3.30 mL, 12.5 mmol, 2.5 equivs.) was dissolved in dry toluene (375 mL) and refluxed. Compound **8** (2.65 g, 4.91 mmol) in dry toluene (90 mL) was added dropwise over 2 h to the solution. After 15 h reaction time the solvent was removed under vacuum and the residue was extracted with hot acetonitrile. The acetonitrile was extracted with hexane and the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate 3:2) to give **9** as an amorphous solid; yield: 1.90 g (4.59 mmol, 94 %); $[\alpha]_D^{20}$: +82° (*c* 0.5, $CHCl_3$).

Allyl 2-Acetamido-2,4-dideoxy-α-D-*xylo*-hexopyranoside (10)

To a solution of **9** (1.76 g, 4.26 mmol) in dry methanol (100 mL) a sodium methanolate solution (7.5 mL) was added at 30 °C and stirred at room temperature for 8 days. After neutralization with Amberlite IR 120, H^+ the solution was filtered and dried to give **10** (989 mg, 4.03 mmol, 95 %) as a colourless solid; mp 153 °C; $[\alpha]_D^{20}$: +239° (*c* 0.8, MeOH).

Allyl 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranoside (15)

Following method 2, *p*NP-Gal (80 mg, 266 μmol) and allyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**11**)[47] (695 mg, 2.66 mmol, 10 equivs.) were incubated in buffer (3.0 mL) with β-galactosidase (25 mg, 1.25 U) to give **15**; Yield: 75.1 mg (177 μmol, 67 %).

Following method 3, *p*NP-Gal (28.8 mg, 96.6 μmol) and **11** (75 mg, 287 μmol, 3 equivs.) were incubated for 50 h in buffer (800 μL) with BSA (40 μg), and β-galactosidase (2.2 μL, 22 U). Dilution with hydrolysis buffer (8 mL), and treatment with β-galactosidase (24 U) from *E. coli* gave **15**; yield: 8.6 mg (20.3 μmol, 21 %).

Following method 4, *p*NP-Gal (21.4 mg, 71.0 μmol) and **11** (55.4 mg, 212 μmol, 3 equivalents) were incubated for 4.5 h in buffer (1.2 mL) with β-galactosidase (20 μL, 420 U). Dilution with hydrolysis buffer (12 mL), and treatment with β-galactosidase from *E. coli* (18 U) gave **15**; yield: 10.3 mg (24.3 μmol, 34 %).

Following method 5, Gal-1-P (14.0 mg, 41.6 μmol) and **11** (13.1 mg, 50.1 μmol, 1.2 equivalents) were incubated in buffer (8.2 mL) with phosphorylase (1.2 mL) gave **15** as a colourless solid; yield: 7.6 mg (17.9 μmol, 43%); mp 224 °C; $[\alpha]_D^{20}$: +104° (*c* 0.5, H_2O); {ref.[49] mp 231 °C; $[\alpha]_D^{20}$: +120° (*c* 1, H_2O)}.

Methyl 2-Acetamido-2-deoxy-3-*O*-(β-D-galacto-pyranosyl)-α-D-galactopyranoside (16)

Following method 2, *p*NP-Gal (57 mg, 189 μmol) and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**12**)[48] (444 mg, 1.89 mmol, 10 equivs.) were incubated in buffer (2.1 mL) with β-galactosidase (18 mg, 902 mU) to give **16**; yield: 49.5 mg (125 μmol, 66%).

Following method 3, *p*NP-Gal (28.8 mg, 96.6 μmol) and **12** (67 mg, 285 μmol, 3 equivs.) were incubated for 50 h in buffer (800 μL) with BSA (40 μg), and β-galactosidase (2.2 μL, 22 U). Dilution with hydrolysis buffer (8 mL), and treatment with β-galactosidase from *E. coli* (24 U) gave **16**; yield: 9.1 mg (22.9 μmol, 24%).

Following method 4, pNP-Gal (21.4 mg, 71.0 μmol) and **12** (50.1 mg, 213 μmol, 3 equivs.) were incubated for 4.5 h in buffer (1.2 mL) with β-galactosidase (20 μL, 420 U). Dilution with hydrolysis buffer (12 mL), and treatment with β-galactosidase from *E. coli* (18 U) gave **16**; yield: 9.3 mg (23.4 μmol, 33%).

Following method 5, Gal-1-P (14.0 mg, 41.6 μmol) and **12** (11.8 mg, 50.1 μmol 1.2 equivs.) were incubated in buffer (8.2 mL) with phosphorylase (1.2 mL) gave **16** as a colourless solid; yield: 7.6 mg (18.4 μmol, 44%); mp 231 °C; $[\alpha]_D^{20}$: +117° (*c* 0.5, H_2O); {ref.[50] mp 235 °C); $[\alpha]_D^{20}$: +128° (*c* 0.38, H_2O)}.

2-Acetamido-1,5-anhydro-2-deoxy-3-*O*-(β-D-galacto-pyranosyl)-D-galactitol (17)

Following method 2, *p*NP-Gal (75.3 mg, 250 μmol) and **13** (513 mg, 2.50 mmol, 10 equivs.) were incubated in buffer (2.8 mL) with β-galactosidase (23.4 mg, 1.17 U) to give **17**; yield: 63.9 mg (1174 μmol, 70%).

Following method 3, *p*NP-Gal (20.0 mg, 66.4 μmol) and **13** (40.9 mg, 199 μmol, 3 equivs.) were incubated for 50 h in buffer (550 μL) with BSA (28 μg), and β-galactosidase (1.5 μl, 15 U). Dilution with hydrolysis buffer (5.5 mL), and treatment with β-galactosidase from *E. coli* (16 U) gave **17**; yield: 4.6 mg (12.5 μmol, 19%).

Following method 4, *p*NP-Gal (15.1 mg, 50.1 μmol) and **13** (30.9 mg, 151 μmol, 3 equivs.) were incubated for 4.5 h in buffer (850 μL) with β-galactosidase (15 μL, 315 U). Dilution with hydrolysis buffer (8.5 mL), and treatment with β-galactosidase from *E. coli* (12.5 U) gave **17**; yield: 5.7 mg (15.5 μmol, 31%).

Following method 5, Gal-1-P (14.0 mg, 41.6 μmol) and **13** (10.3 mg, 50.2 μmol 1.2 equivs.) were incubated in buffer (8.2 mL) with phosphorylase (1.2 mL) to give **17** as a colourless, amorphous solid; yield: 6.3 mg (17.1 μmol, 41%); $[\alpha]_D^{20}$: +63°(*c* 0.2, DMSO/MeOH 1:1).

Allyl 2-Acetamido-2,4-dideoxy-3-*O*-(β-D-galacto-pyranosyl)-α-D-xylo-hexopyranoside (18)

Following method 2, *p*NP-Gal (80 mg, 266 μmol) and **14** (650 mg, 2.65 mmol, 10 equivs.) were incubated in buffer (3 mL) with β-galactosidase (25 mg, 1.25 U) to give **18**; yield: 72.8 mg (179 μmol, 67%).

Following method 3, *p*NP-Gal (20.0 mg, 66.4 μmol) and **14** (49.0 mg, 200 μmol, 3 equivalents) were incubated for 50 h in buffer (600 μL) with BSA (25 μg), and β-galactosidase (1.5 μL, 15 U) to give **18**; yield: 6.0 mg (14.7 μmol, 22%).

Following method 4, *p*NP-Gal (24.1 mg, 80.0 μmol) and **14** (59 mg, 241 μmol, 3 equivalents) were incubated for 4.5 h in buffer (1.4 mL) with β-galactosidase (22 μL, 462 U) to give **18**; yield: 12.0 mg (29.5 μmol, 37%).

Following method 5, Gal-1-P (15.0 mg, 44.6 μmol) and **14** (13 mg, 53 μmol 1.2 equivalents) were incubated in buffer (8.7 mL) with phosphorylase (1.3 mL) to give **18** as an amorphous, colourless solid; yield: 7.4 mg (18.2 μmol, 41%); $[\alpha]_D^{20}$: +42° (*c* 0.1, H_2O).

Allyl (5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic Acid)-(2–3)-(β-D-galactopyranosyl)-(1–3)-2-acetamido-2-deoxy-α-D-galactopyranoside (19)

Following method 1, acceptor disaccharide **15** (15 mg, 35 μmol) and *p*NP-αNeu5 Ac (11 mg, 35 μmol) were incubated with TS for 8 h to give **19** as a colourless solid; yield: 12 mg (16 μmol, 46%); $[\alpha]_{546}^{20}$: +23° (*c* 0.05, H_2O).

Methyl (5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic Acid)-(2–3)-(β-D-galactopyranosyl)-(1–3)-2-acetamido-2-deoxy-α-D-galactopyranoside (20)

Following method 1, acceptor disaccharide **16** (15.5 mg, 32 μmol) and *p*NP-αNeu5 Ac (10.4 mg, 37 μmol) were incubated with TS for 8 h to give **20** as a colourless solid; yield: 8 mg (11 μmol, 30%); mp 181 °C; $[\alpha]_{546}^{20}$: +26° (*c* 0.05, H_2O).

(5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic Acid)-(2–3)-(β-D-galacto-pyranosyl-2-acetamido-1,5-anhydro-2-deoxy-D-galactitol (21)

Following method 1, acceptor disaccharide **17** (16.4 mg, 40 μmol) and *p*NP-αNeu5 Ac (10 mg, 32 μmol) were incubated with TS for 8 h to give **21** as a colourless solid; yield: 13 mg (19 μmol, 44%); $C_{25}H_{47}N_2O_{19}$: 658.62; mp 183 °C; $[\alpha]_{546}^{20}$: +96° (*c* 0.05, H_2O).

Allyl (5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic Acid)-(2–3)-(β-D-galactopyranosyl)-2-acetamido-2,4-dideoxy-α-D-xylo-hexopyranoside (22)

Following method 1, acceptor disaccharide **18** (20 mg, 49 μmol) and *p*NP-αNeu5 Ac (15 mg, 48 μmol) were incubated with TS for 8 h to give **22** as a colourless solid; yield: 14.8 mg (21 μmol, 43 %); mp 172 °C; $[\alpha]_{546}^{20}$: +44° (*c* 0.05, H_2O).

Allyl (5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic Acid)-(2–3)-(α-L-arabinopyranosyl)-(1–3)-2-acetamido-2-deoxy-α-D-galactopyranoside (26)

Following method 1, acceptor disaccharide allyl 2-acetamido-2-deoxy-3-*O*-(α-L-arabinopyranosyl)-α-D-galactopyranoside (**23**)[46] (16 mg, 40 μmol) and *p*NP-αNeu5 Ac (13 mg, 42 μmol) were incubated with TS for 8 h to give **26** as a colourless solid; yield: 16 mg (23 μmol, 46 %); $C_{27}H_{45}N_2O_{19}$: 684.66; mp 167 °C; $[\alpha]_{546}^{20}$: +40° (*c* 0.05, H_2O).

Allyl (5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic Acid)-(2–3)-(α-D-fucopyranosyl)-(1–3)-2-acetamido-2-deoxy-α-D-galactopyranoside (27)

Following method 1, acceptor disaccharide allyl 2-acetamido-2-deoxy-3-*O*-(α-D-fucopyranosyl)-α-D-galactopyranoside (**24**)[46] (13 mg, 32 μmol) and *p*NP-αNeu5 Ac (10 mg, 32 μmol) were incubated with TS for 8 h to give **27** as a colourless solid; yield: 7 mg (10 μmol, 30 %); $C_{28}H_{47}N_2O_{19}$: 698.42; mp 170 °C; $[\alpha]_{546}^{20}$: + 62° (*c* 0.05, H_2O).

Allyl (5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic Acid)-(2–3)-(2-deoxy-β-D-lyxo-hexapyranosyl)-(1–3)-2-acetamido-2-deoxy-α-D-galactopyranoside (28)

Following method 1, acceptor disaccharide allyl 2-acetamido-2-deoxy-3-*O*-(2-deoxy-β-D-*lyxo*-hexopyranosyl)-α-D-galactopyranoside (**25**)[46] (15 mg, 36 μmol) and *p*NP-αNeu5 Ac (13 mg, 42 μmol) were incubated with TS for 8 h to give **28** as a colourless solid; yield: 11 mg (15 μmol, 43 %); mp 179 °C; $[\alpha]_{546}^{20}$: +32° (*c* 0.05, H_2O).

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