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A Highly Selective Enzymatic Synthesis of Galactosides from Lactose

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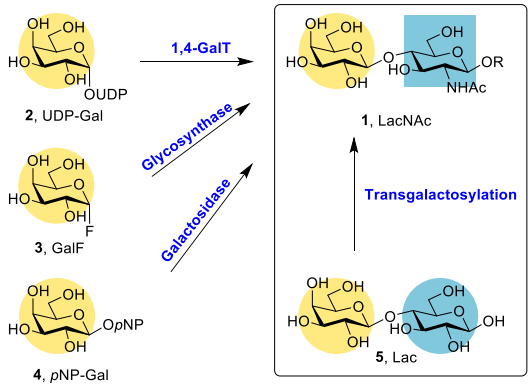
ABSTRACT: The regio- and stereoselective synthesis of galactosides by galactosyltransferases is widely used for the fabrication of complex oligosaccharides and glycoconjugates such as glycoproteins, glycolipids and human milk oligosaccharides. However, a major drawback remains the requirement for expensive cofactor UDP-galactose as the galactosyl donor. Herein we report a biocatalytic route for the synthesis of galactosides directly from lactose, an inexpensive starting material, *via* generation of UDP-galactose as an intermediate. Utilising a fast and sensitive screening method based on imidazolium tagged probes, we report unprecedented promiscuous activity of bacterial β1,4-galactosyltransferases to catalyse the transgalactosylation from lactose to *N*-acetylglucosamine derivatives in the presence of catalytic amounts of UDP. The process is demonstrated by the preparative scale synthesis of *p*NP-β-LacNAc from lactose using β1,4-galactosyltransferase NmLgtB-B as the only biocatalyst. Furthermore, NmLgtB-B is able to be used in combination with α1,4-galactosyltransferase in a one-pot system to assemble a trisaccharide directly from lactose.

Galactosides are among the most abundant glycans in the mammalian glycome and generally biosynthesised by Leloir-galactosyltransferases. In particular, *N*-acetyllactosamine (LacNAc) is a common core glycan motif (Type1 and Type 2 glycans) in free oligosaccharides, glycoproteins and glycolipids. Galactosides, including LacNAc, are important constituents of human milk oligosaccharides, which have great health benefits for infants.[1,2] Sialylated and fucosylated analogues of LacNAc such as sialyl Lewis^X have been described as ligands of various lectins, such as galectins and selectins.[3] The demand of such oligosaccharides for biological investigation and commercialisation as additives to formula milk has increased considerably over the last decade and synthetic methods employing biocatalysts are very attractive compared to multi-step chemical strategies.[4–10] Another important galactoside is Globotriose (Gb3 antigen), usually presented in glycolipids, which play a significant role in pathogen infections[11] as ligands of verotoxin in pathogenic *Escherichia coli*, leading to dysentery and hemorrhagic colitis.[12]

The central role of galactosides in these bioactive oligosaccharides, in particular LacNAc (**1**), has prompted the development of several enzymatic synthetic strategies (Scheme 1). Key to all is the activation of the galactose anomeric centre, since direct glycosidic bond formation from free reducing sugars is unfavourable. In biosynthesis, UDP-galactose (**2**) is commonly utilised as the activated substrate by a wide range of galactosyltransferases, but the cost of this substrate can be prohibitive in large scale synthesis.

Scheme 1. Enzymatic approaches for LacNAc derivative (**1**) synthesis using GlcNAc-R as acceptor and **2-5** as donor substrates.

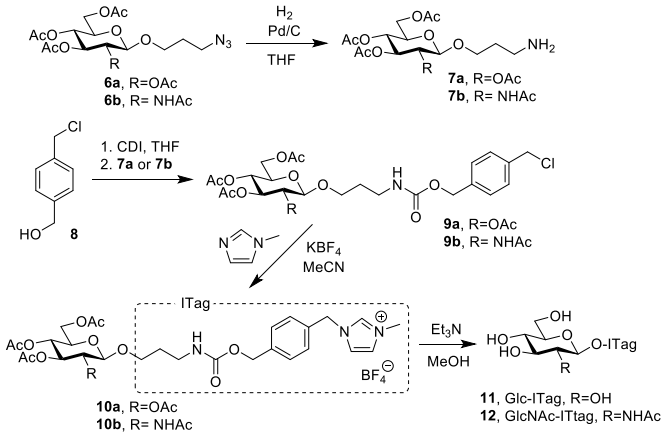


Some elegant alternative synthetic approaches have been developed using activated substrates such as galactosyl fluoride (**3**) [13] and *p*NP-galactose (**4**).[14] UDP-galactose (**2**) can also be regenerated using a multienzyme system either from galactose or sucrose, which

adds additional steps and potential side-products.[15,16] It has been recognised that the most cost-efficient galactosyl donor would be lactose (**5**), a waste product of the cheese industry that is produced at 6 million ton scale every year. So far, the use of lactose (**5**) as substrate has been limited to galactosidases, which have some inherent transgalactosylation activity, but show generally a limited range of selectivity and low yield.[17,18]

These findings prompted us to look for biocatalysts that would be able to generate the universal donor UDP-galactose (**2**) directly *in situ* from lactose (**5**) and UDP without any further cofactor. In general, Leloir transferases are not able to catalyse this reaction. However, the reversible catalytic activity of natural product glycosyltransferases has been described,[19] and was employed to synthesise a wealth of different nucleotide sugars in the presence of nucleotides.[20] In addition, α1,4-galactosyltransferase (LgtC) from *Neisseria meningitidis* and bifunctional α2,3/2,8-sialyltransferase(CstII) from *Campylobacter jejuni* were found able to transfer galactose and sialic acid from alternative sugar donors (i.e., dNP-β-Gal and *p*NP-α-Neu5Ac) to acceptors in the presence of the corresponding nucleotides (i.e., UDP and CMP respectively).[21] Two bacterial α2,6-sialyltransferases were exploited as specific α2,6-sialidases based on their reverse activity.[22,23] Furthermore, glycosyl transfer catalysed by β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) was also reversible.[24] However, to the best of our knowledge there is no report on galactosyltransferases exhibiting reverse transfer from cheaply available lactose.

Scheme 2. Chemical synthesis of Glc-ITag (**11**) and GlcNAc-ITag (**12**).



For the initial screening experiments of transgalactosylation activity, it was important to develop a fast and robust assay that would detect even weak galactose transfer activity in the presence of high excess of lactose **5.** Our previous work had shown that sugar acceptors tagged with imidazolium-based probes (I-Tags) allow for the monitoring of glycosylation reactions by mass spectrometry (MALDI-ToF),[25–28] even in complex mixtures.[29] The cationic I-Tag generates a strong MS signal that dominates the ionisation of the analytes[28] and allows for a reasonable estimate of overall yields by measurement of starting material and product peaks.

For the purpose of the present study, a new class of benzyl carbamate-containing I-Tagged glucosides of Glc (**11**) and GlcNAc (**12**) were chemically synthesised from 1-azidopropyl (**6a**)[30] and (**6b**)[27] in 3 steps, giving 18% and 21% overall yield respectively (Scheme 2; See supporting information for further details). As expected, MALDI-ToF spectra of Glc-ITag (**11**) and GlcNAc-ITag (**12**) provided strong peaks with expected mass in aqueous and buffer solutions (Figure 1). Furthermore, when both I-Tagged substrates were treated with a galactosyltransferase (NmLgtB-A) and UDP-Gal (**2**) the glycosylation products could be clearly observed by MALDI-ToF without any further purification (Figure 1), providing an excellent basis for further screening studies.

The I-Tag methodology was next used to screen galactosyltransferase activity from lactose (**5**) instead of UDP-Gal (**2**). Three bacterial β1,4-galactosyltransferases were cloned from *Neisseria meningitidis* serogroup A strain Z2491(NmLgtB-A), *Neisseria meningitidis* serogroup B strain MC58 (NmLgtB-B)[8] and *Neisseria meningitidis* (NmLgtH). NmLgtB-A and NmLgtB-B are homologous proteins from different strains (92% identity), while NmLgtH shows 71% and 72% identity to NmLgtB-A and NmLgtB-B, respectively, as shown by the amino acid sequence alignment (Supporting information, Figure S2). All three proteins were produced recombinantly in *E. coli* BL21 (DE3) with an N-terminal His_6-tag and purified by affinity chromatography. In the first instance, the activity of the three β1,4-galactosyltransferases was confirmed by using Glc-ITag (**11**) and GlcNAc-ITag (**12**) as substrates (Scheme 3), although LgtH displayed only low activity against GlcNAc-ITag (**12**) (data not shown).

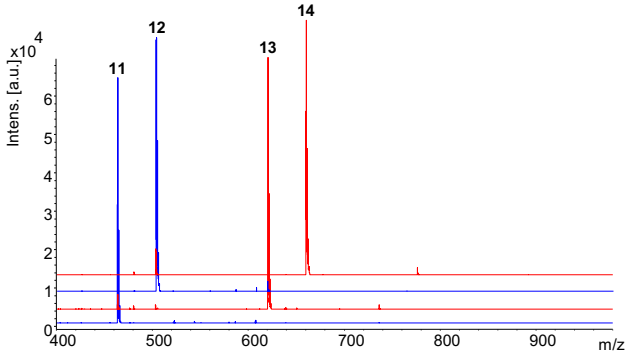
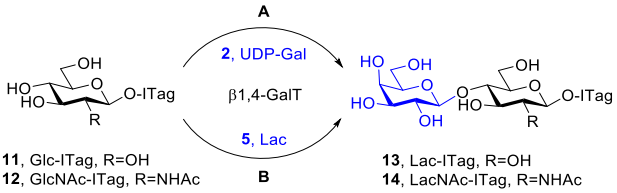


Figure 1. MALDI-ToF mass spectra of ITag substrates (**11** and **12,** blue traces) and their biotransformation products (**13** and **14**, red traces). Glc-ITag **11** $[M]^+$ = 466, GlcNAc-ITag **12** $[M]^+$ = 507, Lac-ITag **13** $[M]^+$ = 628 , and LacNAc-ITag **14** $[M]^+$ = 669.

Having established a sensitive MS-based assay on I-Tagged substrates, lactose (**5**) was tested as galactose donor in the presence of UDP (Scheme 3). Given that the equilibrium between lactose and UDP-Gal would be expected to be unfavourable towards product, UDP-Gal generation was monitored by coupling the reactions with subsequent galactose transfer to the I-Tagged acceptor substrates **11** and **12** (Scheme 3), which would result in transfer of galactose from lactose to substrate *via in situ* formation of UDP-Gal (**2**). Rewardingly, formation of Lac-ITag (**13**) and LacNAc-ITag (**14**) from **11** and **12** (Scheme 3) could be detected by MALDI-ToF spectrometry in the NmLgtB-B catalysed reactions, while NmLgtB-A afforded only a very low conversion and NmLgtH no conversion at all (Supporting information, from Figure S3 to S8). Therefore, NmLgtB-B was used in subsequent experiments.

Scheme 3. Investigation of galactosyltransferase activity with I-Tag acceptor substrates **11** and **12** using A: UDP-Gal (**2**) or B: lactose (**5**) as sugar donor substrates.



The scope of galactose donor substrates beyond lactose was also tested using *p*NP-β-Lac, *p*NP-α-Gal, *p*NP-β-Gal and LacNAc (**1**) as galactose donors in the presence of UDP. Galactose transfer was also observed in reactions containing *p*NP-β-Lac and LacNAc (Supporting information, Figure S9 and S10). However, since lactose (**5**) is very inexpensive and easily available, it was subsequently used as a preferable galactose donor.

The optimal reaction conditions for the transgalactosylation activity of NmLgtB-B were explored by studying the effects of pH, UDP and lactose (**5**) concentrations on reaction yields (Figure 2), using *p*NP-β-GlcNAc (**15**) as acceptor. Interestingly, with NmLgtB-B higher conversions could be achieved in acidic environment, with a maximum around pH 5.0 (Figure 2A).

In the absence of UDP no conversion was observed, suggesting that UDP is required for the transfer reaction and providing the first mechanistic clue that the reactions would proceed *via* UDP-galactose (**2**). Product formation increased gradually when the concentration of UDP was increased, with a plateau reached at 2 mM (Figure 2B). The yield of reaction was measured at different lactose (**5**) concentrations (Figure 2C), establishing that substrate concentrations above 20mM did not afford significant increases in the conversion.

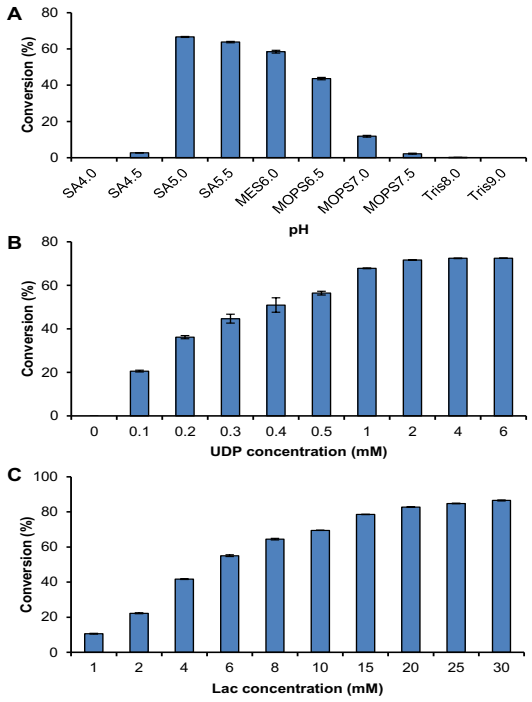
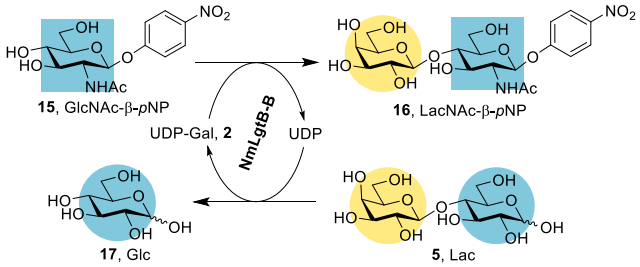


Figure 2. Conversion of *p*NP-β-GlcNAc (**15**) to **16** dependence on A: pH; B: UDP concentration and C: lactose (**5**) concentration.

The formation of UDP-Gal (**2**) as an intermediate was also verified directly by high resolution mass spectrometry of the crude incubation mixture. In a reaction mixture containing lactose (**5**), UDP and NmLgtB-B in acetate buffer pH5.0 incubated at 37°C overnight it was possible to detect the presence of UDP-Gal (**2**), while a negative control without enzyme did not show the diagnostic peaks (Supporting information, Figure S11).

Scheme 4. Preparative synthesis of LacNAc-β-*p*NP(**16**).

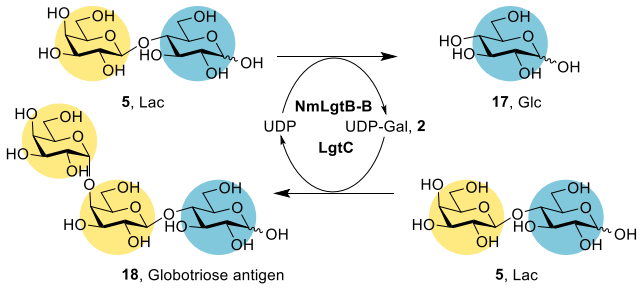


The practical application of galactosylation from lactose (**5**) was demonstrated by the preparative scale synthesis of LacNAc-β-*p*NP (**16**) (Scheme 4). GlcNAc-β-*p*NP (**15;** 51μmole), lactose (**5**; 1.0mmole) and UDP (112 μmole) were incubated with NmLgtB-B at 37°C for 14 hours, which gave 90% conversion as measured by HPLC (Supporting information, Figure S13). The

unreacted GlcNAc-β-*p*NP (**15**) was removed by hydrolysis with β-*N*-acetylhexosaminidase, which allowed for the product to be isolated by preparative reverse phase HPLC, yielding LacNAc-β-*p*NP (**16**) in 50% overall yield.

Finally, to extend this technology, we explored if the transient UDP-galactose (**2**) generated from lactose (**5**) could be used by a second galactosyltransferase in the same reaction mixture. Therefore, a α1,4-galactosyltransferase (LgtC) which had been shown to be responsible for the formation of the globotriose antigen **18** was used in a one-pot two enzymes system as shown in Scheme 5. Formation of a trisaccharide assigned as globotriose (**18**) using lactose (**5**) both as acceptor and donor substrate was indeed observed using MALDI-ToF mass spectrometry (Supporting information, Figure S15).

Scheme 5. One-pot two enzymes synthesis of globotriose **18**.



In conclusion, the reversibility of the catalytic activity of β1,4-galactosyltransferases, in particular NmLgtB-B, was demonstrated for the first time by utilising novel imidazolium tagged substrates. This reversibility allowed for the *in situ* formation of UDP-Gal (**2**) from inexpensive lactose (**5**) in the presence of UDP. By adding a further acceptor substrate the overall transfer of galactose from lactose (**5**) to acceptor substrates **11**, **12** and **15** could be observed. The practical applicability of this simple and cost-efficient transgalactosylation method was demonstrated by preparative scale synthesis of LacNAc-β-*p*NP (**16**) from lactose using NmLgtB-B as the only biocatalyst. In addition, this UDP-Gal generation system could be coupled with another galactosyltransferase in a one-pot system to form more complex trisaccharides. These results are particularly interesting because lactose **5** is a waste product in the milk industry and the current transglycosylation strategy provides opportunities for converting bio waste into high value products.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization of new compounds. This material is available free of charge via the internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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