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A β-1,4-Galactosyltransferase from *Helicobacter pylori* is an Efficient and Versatile Biocatalyst Displaying a Novel Activity for Thioglycoside Synthesis

Darius-Jean Namdjou,[a] Hong-Ming Chen,[b] Evguenii Vinogradov,[a] Denis Brochu,[a] Stephen G. Withers,[b] and Warren W. Wakarchuk*[a]

Helicobacter pylori *is a highly persistent and common pathogen in humans. It is the causative agent of chronic gastritis and its further stages. HP0826 is the β-1,4-galactosyltransferase involved in the biosynthesis of the LPS O-chain backbone of* H. pylori. *Though it was first cloned nearly a decade ago, there are surprisingly limited data about the characteristics of HP0826, especially given its prominent role in* H. pylori *pathogenicity. We here demonstrate that HP0826 is a highly efficient and promiscuous biocatalyst. We have exploited two novel enzymatic activities for the quantitative synthesis of the thiodisaccharide Gal-β-S-1,4-GlcNAc-*p*NP as well as Gal-β-1,4-Man-*p*NP. We further show that* Neisseria meningitidis *β-1,4-galactosyltransferases LgtB can be used as an equally efficient catalyst in the latter reaction. Thiodisaccharides have been extensively used in structural biology but can also have therapeutic uses. The Gal-β-1,4-Man linkage is found in the* Leishmania *species LPG backbone disaccharide repeats and cap, which have been associated with vector binding in Leishmaniasis.*

Introduction

Glycosyltransferases have proven to be of great importance in oligosaccharide synthesis. High-level soluble expression of the biocatalyst is often a prerequisite for synthetic work. Mammalian glycosyltransferases are limited in this respect as a sufficient expression level is, in general, only reached in eukaryotic cells. In contrast several bacterial enzymes have been shown to express well in *E. coli*.[1–3] Gram-negative pathogenic bacteria possessing lipooligosaccharides (LOS) or lipopolysaccharides (LPS) which mimic human carbohydrate cell surface structures are a prime source of the relevant glycosyltransferases. *Helicobacter pylori* is such a pathogen as it displays an LPS terminating in fucosylated oligosaccharides mimicking Lewis (Le) antigens. *H. pylori* is the causative agent of gastritis and its later stages involving gastric cancer.[4,5] The role of the LPS Le type structures in the pathogenicity of *H. pylori* is still a matter of lively debate. In recent data the high variability and phase variation of Le antigens has been shown to modulate host adaptive immune T-helper cell response, creating an ideal balance of T-helper cells for persistent pathogen colonization.[4] Phase variation of the Le antigens has also been described as a key factor in evasion of host innate immune response.[6] The gene HP0826 coding for the β-1,4-galactosyltransferase (β4GalT, E.C. 2.4.1.22) involved in biosynthesis of the O-chain backbone type 2 *N*-acetyl-lactosamine (LacNAc) and Le structures has been identified and cloned.[7,8] A *H. pylori* strain in which HP0826 has been disrupted produced an O-chain shortened to a single terminal GlcNAc moiety and failed to induce gastritis in mice.[9] Taking all these findings together the β4GalT of *H. pylori* appears to be a good candidate for high-level expression and possible usage as a biocatalyst. It is surprising that nearly a decade after the identification of HP0826 only limited data exists concerning the biosynthetic potential of this galactosyltransferase. Liu and co-workers used the enzyme in an approach with engineered and permeabilized *E. coli* cells to synthesize P1 trisaccharide Galα1,4Galβ1,4GlcNAc. These authors observed a rather modest activity of up to 40 $U L^{-1}$ for the transfer of UDP-Galactose (UDP-Gal) to GlcNAc.[10] In another application human immunoglobulin G (IgG) N-glycans were galactosylated, but no activities were given for this reaction.[11]

In addition to their ease in expression, substrate promiscuity is a further hallmark of bacterial glycosyltransferases and a significant difference compared to the mammalian enzymes.[12] *Neisseria meningitidis* galactosyltransferases LgtB[13] and LgtC[14] as well as several glycosyltransferases from *Campylobacter jejuni*[3] have been studied regarding their acceptor substrate specificity and used for synthetic work employing a wide range of unnatural acceptors. No such characterization of the acceptor specificity of the *H. pylori* β-1,4-galactosyltransferase has been undertaken and so a major synthetic potential has thus remained unexplored.

Thioglycosides have been the focus of ongoing research for many years. Techniques for their synthesis and the most important applications have recently been reviewed.[15] Those compounds in which the oxygen of the glycosidic linkage is

[a] *D.-J. Namdjou, E. Vinogradov, D. Brochu, Prof. W. W. Wakarchuk*
Institute for Biological Sciences, National Research Council Canada
100 Sussex Drive, Ottawa, ON, K1A 0R6 (Canada)
Fax: (+1) 613-941-1327
E-mail: warren.wakarchuk@nrc-cnrc.gc.ca

[b] *H.-M. Chen, Prof. S. G. Withers*
Department of Chemistry, University of British Columbia
2036 Main Mall, Vancouver, BC, V6T 1Z1 (Canada)

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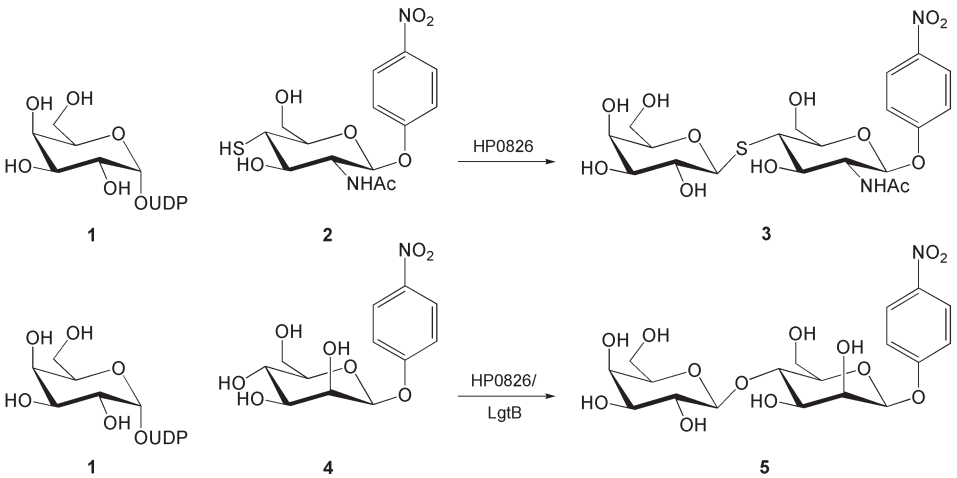
replaced by sulfur have long been known to be resistant to cleavage by glycosidases.[16] A major class of thiosugars are the thiodisaccharides and thiooligosaccharides. Numerous thioglycosides were shown to be competitive inhibitors of glycosidases which are good ligands for crystallographic studies.[17] A significant growth inhibitory effect of thiodisaccharides on cancer cell lines has been observed, indicating their potential as anticancer agents.[18] In addition, Sialyl Le antigen (SLe antigens) mimetics with thio-linked precursors have been described as templates for novel antimetastic agents.[19] Conjugate vaccines consisting of immunogenic carrier proteins and thio-oligosaccharides have been shown to elicit an immune response and have a potential application in immunotherapy.[20]

The synthesis of the relevant thioglycosides has been and is still a domain of the organic chemist.[16,21–23] All these methods involve rather laborious multistep protection group chemistry. In recent years an alternative chemoenzymatic method has evolved employing the use of mutant glycosidases, called thioglycoligases.[24,25] Retaining glycosidases missing the catalytic acid/base amino acid catalyze the reaction of a donor carrying a good leaving group (for example, a dinitrophenyl glycoside) with a strong nucleophile (for example, a thiosugar). Still, both donor and acceptor substrate have to be synthesized chemically. In further work, so-called thioglycosynthases, double mutant glycosidases, have been developed in which the catalytic nucleophile amino acid has been removed. Glycosyl fluorides can also be used as donor substrates which are easier to synthesize than dinitrophenylglycosides. Additionally nucleophile mutants are completely hydrolytically inactive whereas the thioglycoligases show background hydrolysis of the donor substrate.[26] Glycosyltransferases have so far found only very limited use in the synthesis of thiooligosaccharides. This is despite the obvious advantage that the synthesis of a sulfur-containing donor or acceptor substrate is the only chemical step necessary for the transferase-catalyzed formation of a sulfur-containing disaccharide. Whereas there have been reports on the synthesis and glycosyltransferase transfer of 5-thio-containing donor[27,28] and acceptor substrates[29] there have been only two examples of the transferase-catalyzed formation of a sulfur-containing glycosidic bond. A mutant of Cst-II, the α-2,3-sialyltransferase from *Campylobacter jejuni*, identified by high-throughput screening has been shown to transfer *N*-acetyl-neuraminic acid to the synthetic fluorescent acceptor substrate bodipy-3-SH-lactose.[30] Recombinant bovine α1,3-galactosyltransferase is the only native glycosyltransferase found yet for thioglycoside linkage formation and has been used for the transfer of Gal to octyl-β-D-3′-thiolactoside.[31] This approach was generally successful but was hampered by the unexpected formation of a tetrasaccharide product because of a second galactosylation of the intermediate trisaccharide. No galactosyltransferase-mediated formation of a β-S-1,4-linkage has so far been achieved. Given the general importance of thiooligosaccharides and the ubiquity of the Galβ-1,4GlcNAc-linkage in glycoconjugates a transferase able to fill this gap would be a useful synthetic tool.

The Galβ-1,4Man linkage is common in the backbone disaccharide repeats and the neutral caps of lipopolyphosphoglycan (LPG) from *Leishmania* species. These parasites are responsible for Leishmaniasis, a disease highly varying in severity in humans including fatal outcome.[32,33] LPG is the major glycoconjugate of the promastigote surface glycocalyx. The number of PG repeats has been associated with conformations enabling and disabling binding of the sandfly (vector) midgut for *Leishmania donovani* and *Leishmania major*.[34] In addition the Gal-containing neutral caps attached to the backbone repeats could be shown to be directly responsible for midgut binding by *Leishmania donovani*.[35] The galactosyltransferase involved in the biosynthesis of the Galβ-1,4Man linkage has not been identified. In order to have the relevant structures readily available for in vitro experiments a GalT capable of formation of the Galβ-1,4Man bond would thus be highly desirable.

In this manuscript we present the high-level expression of the HP0826-encoded β-1,4-galactosyltransferase from *H. pylori* and demonstrate that this enzyme is an efficient and versatile biocatalyst showing pronounced acceptor substrate promiscuity. We exploited this activity for the chemoenzymatic synthesis of 4-nitrophenyl (β-D-galactopyranosyl)-(1→4)-S-2-acetamido-2,4-deoxy-4-thio-β-D-glucopyranoside (Gal-β-S-1,4-GlcNAc-β-*p*NP; **3**) from UDP-Gal (**1**) and 4-nitrophenyl 2-acetamido-2-deoxy-4-thio-β-D-glucopyranoside (*p*NP-4S-β-GlcNAc (**2**), Scheme 1). To our knowledge this is the first example of the galactosyltransferase-mediated formation of the Gal-β-S-1,4-GlcNAc bond. We further utilized the promiscuous acceptor specificity of the HP0826 for the synthesis of 4-nitrophenyl (β-D-galactopyranosyl)-(1→4)-mannopyranoside (Galβ-1,4Man-β-*p*NP; **5**) using 4-nitrophenyl β-D-mannopyranoside (*p*NP-β-



Scheme 1. A) HP0826 catalyzed synthesis of Gal-β-S-1,4-GlcNAc-β-*p*NP (**5**). B) HP0826 or LgtB catalyzed synthesis of Gal-β-1,4-Man-β-*p*NP (**6**).

Man; **4**) as acceptor substrate. We also show catalysis of the same reaction with another bacterial galactosyltransferase (Scheme 1). HP0826 and LgtB are up to now the only galactosyltransferases mediating the formation of the Galβ-1,4Man bond. NMR data for the synthesis with HP0826 show that both unusual bonds are formed with total regio- and stereoselectivity. We have kinetically characterized HP0826 for the transfer of Gal to *p*NP-β-GlcNAc, *p*NP-4-S-β-GlcNAc, and *p*NP-β-Man.

Results and Discussion

Soluble high-level expression and purification of HP0826

HP0826 was expressed as full-length enzyme in *E. coli* by using the expression vector pHP21 derived from pCWori+.[38] The vector pCWori+ has been used for soluble expression of several bacterial glycosyltransferases including LgtB.[1,3] SDS-PAGE analysis showed that HP0826 is found in both the supernatant and the pellet fractions, but that most of the activity is found in the supernatant fraction (Figure 1). We determined

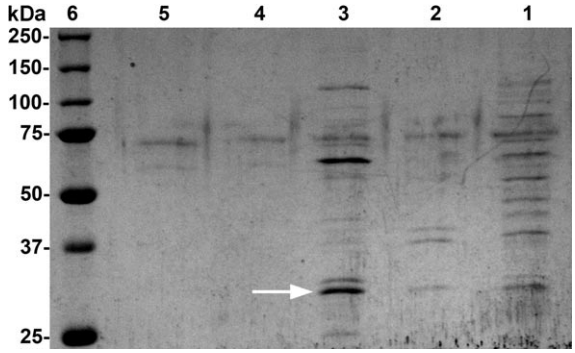


Figure 1. SDS-PAGE analysis of the expression and purification of HP0826, lane 1: cell extract supernatant; lane 2: cell extract pellet; lane 3: anion-exchange flow-through fraction A6; lane 4: elution fraction C11; lane 5: elution fraction E1; lane 6: molecular weight marker (all lanes containing 5 μg of protein). Arrow indicates the HP0826 protein.

1600 U L^{-1} or 160 U g^{-1} activity in the crude extract using acceptor substrate *p*NP-β-GlcNAc. In comparison, a recently published study of three different constructs of human β4GalT-1 expressed in *E. coli* showed a maximum activity of 27 U g^{-1} in the crude extract. In these studies the related acceptor substrate benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside was used.[44] Results for the respective specific activities are a further example of the superiority of the expression of bacterial enzymes in *E. coli*.

Though it has been shown that synthetic work is generally possible with a crude preparation of HP0826,[11] we opted for a partial purification of the enzyme in order to have a better defined enzyme solution and a lower risk of contaminating enzymatic activities, especially in view of our work with unusual acceptor substrates such as *p*NP-4S-β-GlcNAc. We established an easy one-step anion-exchange procedure leading to significant enrichment of the protein (Figure 1) and an approximately threefold increase in specific activity. No contaminating activities were detected in enzymatic assays with negative controls (without donor or acceptor), and the obtained purity proved to be sufficient for all work within this publication.

Biochemical characterization of HP0826

HP0826 belongs to the carbohydrate active enzyme (CAZy) family GT25 of inverting glycosyltransferases. It shows only modest sequence identity to other family members, 39% with lex2B, a glucosyltransferase from *Haemophilus influenzae* and 16% with LgtB, the best studied β4GalT of this family. Given these numbers and a marked absence of data, we set out to explore the basic biochemical characteristics of HP0826, with the primary purpose of determining the optimum conditions for biocatalysis. We examined the pH optimum as well as the optimal metal cofactor, if any. We found the best activity in HEPES/NaOH buffer pH 7.2 (Figure 2A). This pH value is similar

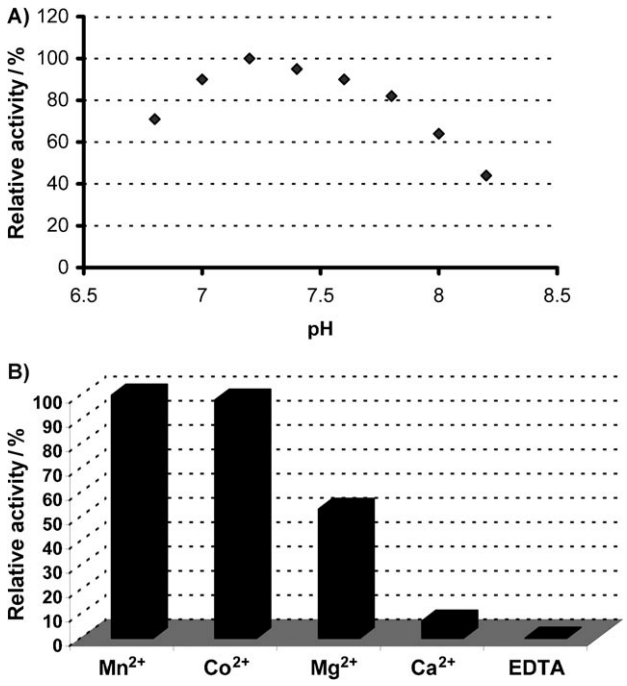


Figure 2. A) HP0826 pH activity profile (100% activity corresponds to 290 U g^{-1}). B) Metal ion dependence of HP0826 activity (100% activity corresponds to 290 U g^{-1}).

to reported pH optima for other β4GalTs, for example, human β4GalT-1.[44] There is a motif EDD associated with catalysis and metal ion binding in the HP0826 sequence, which has been described to be similar to the better known glycosyltransferase motif DXD.[45] We therefore expected a dependence of the enzyme activity on divalent metal cations, most likely Mn^{2+}. Our experimental data confirm these assumptions (Figure 2B). Whereas all metal ions used activated the enzyme, there was no activity in a control reaction containing EDTA (Figure 2B). Selectivity towards metal ions appears to be low, though. Mg^{2+} (53%) and especially Co^{2+} (98%) show high residual activities when compared to metal ion activation by Mn^{2+}.

Results for bovine GalT (40% residual activity)[46] and human β4GalT-1 (20% residual activity)[44] clearly demonstrate less acceptance by these two mammalian enzymes for metal cations other than Mn^{2+}. HEPES pH 7.2 and metal cofactor Mn^{2+} were used in all further work with HP0826. A check of the donor specificity of HP0826 using UDP-GalNAc (2.8% residual activity compared to UDP-Gal), UDP-Glc (0.2%), and UDP-GlcNAc (0.06%) did not show any uncommon properties. Donor promiscuity at similar residual activity levels has for example, also been shown for the bovine enzyme.[47]

Table 2. Acceptor substrate activity determinations for HP0826.[a]

Acceptor substrate	Specific activity [U L g^{-1}]
*p*NP-GlcNAc	330.5 (100%)
*p*NP-4-S-GlcNAc	2.9 (0.9%)
*p*NP-Glc	80.0 (24.2%)
*p*NP-Man	2.6 (0.8%)
*p*FP-All[b]	–
*p*NP-GalNAc	–
*p*NP-Gal	–
*p*NP-Xyl	1.7 (0.5%)

[a] All acceptors are β-anomers. [b] See the Supporting Information for structure.

Kinetic characterization of HP0826 with acceptor *p*NP-β-GlcNAc

We have determined the kinetic constants for the transfer from UDP-Gal to *p*NP-β-GlcNAc catalyzed by HP0826 (Table 1). We used single substrate variation for endpoint activity measurements which were analyzed by CE.

As we did not deal with a highly purified protein, the values for specific activity and therefore also catalytic efficiency have to be regarded as preliminary results. The most valid comparison with published data is seen in substrate affinities (K_M). Recent studies with the β4GalT from human milk and its human counterpart show that hydrophobic substrates (explicitly *p*NP-β-GlcNAc) are highly preferred acceptor substrates when compared with the enzyme affinity towards free GlcNAc.[44,48] These reports give K_M values from 0.15 to 0.92 mM for *p*NP-β-GlcNAc, with our value of 0.79 mM in the same range. The HP0826 K_M value for UDP-Gal is also similar to the experimental data for the two mammalian enzymes.[44,47]

High substrate affinities are a prerequisite for crystal structure elucidation. Up to now no crystal structure of a member of the CAZy GT25 family has been solved. Given its high expression level and substrate affinities HP0826 might fill this gap and widen our limited understanding of this particular GT family.

Acceptor substrate screening for HP0826

We determined enzyme activity with several *p*NP-glycosides in the transfer of UDP-Gal by HP0826. We systematically examined differences in acceptor sugar stereochemistry compared to the natural acceptor GlcNAc around the carbohydrate ring. Other than GlcNAc 4-epimer GalNAc, activities for Glc, its 2-epimer Man, 3-epimer All, 4-epimer Gal, and 6-deoxy-derivative Xyl were measured. In addition the sulfur-containing acceptor *p*NP-4-S-β-GlcNAc was chosen as a potential acceptor substrate for the galactosyltransferase-mediated synthesis of a thiodisaccharide. Table 2 shows the results for the respective β-anomers. With the exception of *p*NP-α-GlcNAc all tested α-anomers were inactive with HP0826. *p*NP-α-GlcNAc showed 17% residual activity when compared to its β-anomer. The preference for β-anomers has also been reported for other β4GalTs, for example, recently for the bovine enzyme.[48]

HP0826 exhibits a pronounced acceptor promiscuity with the most striking result being the successful transfer of Gal to the 4-SH of *p*NP-4-S-β-GlcNAc. The specific activity of 2.9 U g^{-1} is in the same range as for the accepted Glc-epimers and sufficient for synthetic work. The differences in size (S against O) and electronegativity are seemingly outweighed by the otherwise unchanged stereochemistry of *p*NP-4-S-β-GlcNAc. Further analysis was needed to show if UDP-Gal transfer indeed led to formation of a S-glycosidic bond or if the regiospecifity of HP0826 was changed under the influence of the S-containing acceptor molecule.

Examining the results, *p*NP-Glc is a good acceptor with more than 20% residual activity when compared to the more natural acceptor *p*NP-GlcNAc. More importantly, in view of our desired synthesis of Galβ-1,4Man-β-*p*NP, *p*NP-Man is also a synthetically useful acceptor substrate with 3.3% residual activity in direct comparison to its 2-epimer Glc. Similar values were obtained for *p*NP-Xyl. No activity could be seen for the 3- and 4-epimers. The result for the 4-position is expected as this hydroxyl group is involved in glycoside bond formation. Addition of α-lactalbumin had no influence on activities or product formation. Although, there have been no systematic studies on epimers as acceptors for mammalian galactosyltransferases, recent results for LgtC, the α4GalT from *Neisseria meningitidis* are of important comparative value.[14] This bacterial enzyme's natural acceptor is the Gal moiety of lactose. Gal itself is also an acceptor substrate albeit with only 0.1% catalytic efficiency when compared to Lac. Gal-epimers All, Man, Xyl, and even 4-epimer Glc are all acceptor substrates in the range of the catalytic efficiency for Gal and have been used for synthetic work. It is noteworthy that only reactions with hydrophobic acceptor glycosides, for example, *p*NP, led to regiospecific product formation.

Table 1. Kinetic constants for HP0826 transfer of UDP-Gal to *p*NP-β-GlcNAc.

	UDP-Gal			*p*NP-GlcNAc		
	$V_{max\ app}$ [U g^{-1}]	$K_{M\ app}$ [mM]	$V_{max\ app}/K_{M\ app} \times 10^{-3}$	$V_{max\ app}$ [U g^{-1}]	$K_{M\ app}$ [mM]	$V_{max\ app}/K_{M\ app} \times 10^{-3}$
HP0826	351.7 ± 3.8	0.27 ± 0.02	1.30	487.4 ± 0.01	0.79 ± 0.07	0.62

Investigations with other bacterial glycosyltransferases are needed to show if successful transfer to substrates with partially inverted stereochemistry is a common characteristic of this enzyme class. The fact that in contrast to HP0826, LgtC is a retaining GalT belonging to CAZy family eight may hint in this direction.

All activities (except for the reaction with the natural acceptor) for HP0826 were confirmed by CE-ESI-MS analysis of disaccharide formation (see the Supporting Information). Product yields for the transfer of UDP-Gal to *p*NP-Glc and *p*NP-Xyl were determined as 84 and 30%, respectively. As the formation of Galβ1,4-Glc is a long-established activity of mammalian lactose synthase we did not exploit the reaction of HP0826 with *p*NP-Glc. This activity could be of use in applications when the presence of α-lactalbumin, essential for the reaction with mammalian galactosyltransferases, is unwanted. Likewise, the low yield for the reaction with *p*NP-Xyl did not invite further examination.

Synthesis of Gal-β-S-1,4-GlcNAc-β-*p*NP (5) with HP0826

Transfer of UDP-Gal to *p*NP-4-S-GlcNAc by HP0826 is a novel glycosyltransferase reaction. We did not see any transfer to the acceptor *p*NP-4-S-GlcNAc using human β4GalT-1. LgtB showed an activity level insufficient for synthetic work (ca. 0.2 mU mg^{-1}, less than 10% residual activity when compared to HP0826). Thus only the HP0826 catalyzed reaction was explored in more detail. To determine the reaction yield and duration we recorded a yield–time curve (Figure 3). Product formation was unexpectedly smooth, and a quantitative conversion of *p*NP-4-S-GlcNAc could be obtained after 5 h. 150 mU of HP0826 were sufficient for conversion of *p*NP-4-S-GlcNAc on the 0.5 mg scale. After being purified on a Sep Pak reversed-phase column, the product was isolated in 88% yield. NMR analysis revealed that the sole product formed was Gal-β-S-1,4-GlcNAc-β-*p*NP (Supporting Information). We thus demonstrate for the first time the galactosyltransferase-mediated formation of the Gal-β-S-1,4-GlcNAc bond. In the only other reported formation of a thioglycosidic bond by the action of a galactosyltransferase (α1,3), Rich and co-workers reported a reaction duration of two days and a use of at least ten times more enzyme to reach a similarly excellent yield.[31] A recent thioglycoligase synthesis of **5** led to only 47% yield after purification.[25] Thus we not only present this unique glycosyltransferase activity but also show that it can be utilized in a highly efficient synthetic process. This activity should also enable an easy scale-up of the reaction. The only drawback from a synthetic point of view is the high concentration of the expensive donor sugar UDP-Gal (20 mM) necessary to reach complete conversion of *p*NP-4-S-GlcNAc (1.5 mM). Reaction yield is limited to 90% and less when lower concentrations of UDP-Gal are used (data not shown). This problem should be rather easily solvable by utilization of the cheaper donor substrate UDP-Glc and in situ conversion to UDP-Gal by a UDP-Glc 4′-epimerase (EC 5.1.3.2). There are numerous examples of this epimerase application in the literature, and also of epimerase/galactosyltransferase fusion proteins, as has been demonstrated for LgtB.[13] As an even more sophisticated option, a nucleotide-sugar-regeneration cycle involving the epimerase may be used as shown for the synthesis of LacNAc.[49,50] In this context, preliminary data show that HP0826 exhibits total donor specificity in the transfer to *p*NP-4-S-GlcNAc, no transfer product is formed when UDP-Glc is used. Further, the presence of up to 20 mM UDP-Glc does not interfere with the transfer reaction of UDP-Gal to *p*NP-4-S-GlcNAc.

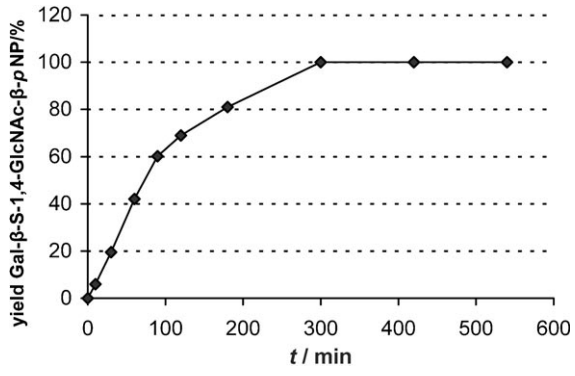


Figure 3. Yield–time curve for the HP0826 catalyzed synthesis of Gal-β-S-1,4-GlcNAc-β-*p*NP (**5**).

We characterized the formation of Gal-β-S-1,4-GlcNAc-β-*p*NP kinetically. When varying the concentration of the acceptor substrate up to its limit of solubility, no saturation level in specific activity could be observed. Therefore no kinetic constants could be obtained. A decrease in affinity is expected for a modified substrate. Given a K_M of 0.79 mM for *p*NP-β-GlcNAc and a maximum possible assay concentration of 2.4 mM for *p*NP-4-S-GlcNAc (limit of solubility) it is not surprising that saturation could not be reached.

Possible applications of the synthesized thiodisaccharide are given by the general importance of this compound class as outlined in the introduction. Although we could show that Gal-β-S-1,4-GlcNAc-β-*p*NP is not cleaved by the β1,4-galactosidase from *Streptococcus pneumoniae* (EC 3.2.1.23), it has already been demonstrated that this particular disaccharide is a weak inhibitor of β-galactosidases from the three major families.[25] It is as such not useful for glycosidase crystal structure analysis. Nevertheless, the unique thioglycoside formation catalyzed by HP0826 should be an important tool for the general study of glycosyltransferase binding and catalysis using thiosugar acceptor substrates. In this context crystal structure analysis and also saturation transfer difference (STD) NMR experiments[51,52] should give valuable insights into this reaction.

Synthesis of Galβ-1,4Man-β-*p*NP (6) with HP0826 and LgtB

In HP0826 we have already identified a possible candidate for a galactosyltranferase-mediated synthesis of the Leishmaniasis-associated structure Galβ-1,4Man. Although we did not see any activity with *p*NP-β-Man and human β4GalT-1, the other GT-25 enzyme LgtB was found to catalyze Gal-transfer to the acceptor substrate. Because of these results we probed both HP0826 and LgtB for this reaction. The yield–time curve

(Figure 4) shows that both enzymes can be used as either of them will form the product in quantitative yield in about 24 h. We used the same protein amount in each assay to allow for a

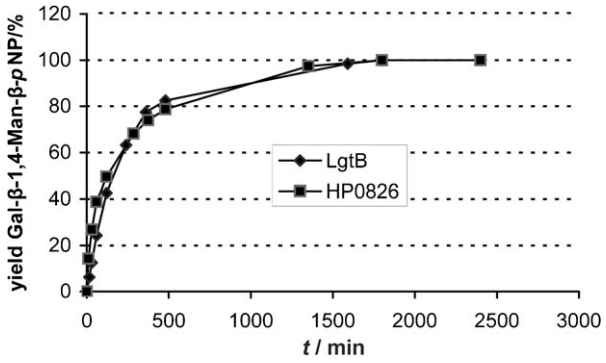


Figure 4. Yield–time curve for the HP0826 or LgtB catalyzed synthesis of Gal-β-1,4-Man-β-*p*NP (**6**).

valid comparison. HP0826 was employed for a synthesis on the 1.5 mg scale. NMR analysis showed that the product was indeed Galβ-1,4Man-β-*p*NP, which was formed with complete stereo- and regioselectivity. The product of the LgtB reaction was also identified as Galβ-1,4Man-β-*p*NP as it showed an identical CE migration time to the HP0826 product and was cleavable by the β1,4-galactosidase from *Streptococcus pneumoniae*. A kinetic examination of both HP0826 and LgtB-catalyzed disaccharide formation (variation of the acceptor substrate concentration) shows a striking contrast (Figure 5). In the case of

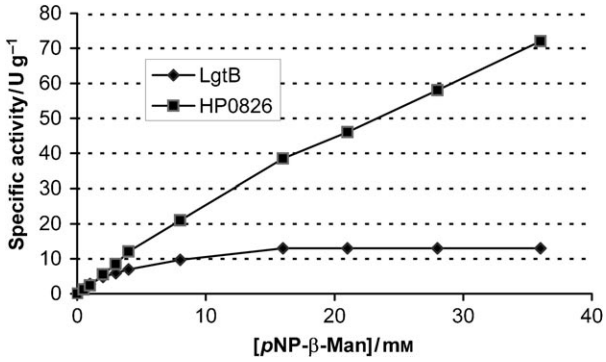


Figure 5. Dependence of specific activity on acceptor substrate concentration for the transfer of UDP-Gal to *p*NP-β-Man (**4**) by HP0826 or LgtB.

HP0826 there is no saturation in specific activity up to the solubility limit of *p*NP-β-Man. For LgtB we found classical Michaelis–Menten behavior and were able to determine kinetic constants of $K_M = 4.8$ mM and $V_{max} = 15.8\ U\,g^{-1}$. *p*NP-β-Man is the preferred acceptor substrate for LgtB, with about 1.4 times higher activity than *p*NP-β-GlcNAc in the transfer of UDP-Gal.

It is quite striking that these 2 GT-25 enzymes show very different behavior with these monosaccharide acceptors. Clearly the catalytic efficiency of HP0826 is far higher than LgtB for the *p*NP-β-GlcNAc and *p*NP-β-Man acceptors, even though both can be used for preparative synthesis. It suggests that there is no product inhibition for these acceptors, and that the catalytic center of these enzymes accommodates these acceptors in very different ways. In future work we plan to examine the details of how these related enzymes discriminate these kinds of synthetic acceptors and will try to further exploit them for the synthesis of useful galactosides.

Conclusions

In this paper we have demonstrated the high catalytic potential of HP0826 by the first galactosyltransferase-mediated synthesis of the thioglycosidic bond Galβ-S-1,4-GlcNAc. LgtB and HP0826 are also the first examples of β4GalTs for the synthesis of the Galβ-1,4Man linkage. Further work will focus on purifying HP0826 to homogeneity in an effort to crystallize this unusually versatile enzyme as the first member of its family. A directed-evolution approach will be applied in order to further widen the substrate promiscuity and thus possible applications of HP0826.

Experimental Section

UDP-Gal was from Calbiochem, USA. UDP-*N*-acetyl-galactosamine (UDP-GalNAc), UDP-glucose (UDP-Glc), and UDP-*N*-acetyl-glucosamine (UDP-GlcNAc) were from Sigma–Aldrich, USA. α and β anomers of 4-nitrophenyl D-galactopyranoside (*p*NP-α-Gal and *p*NP-β-Gal), 4-nitrophenyl 2-acetamido-2-deoxy-D-galactopyranoside (*p*NP-α-GalNAc and *p*NP-β-GalNAc), 4-nitrophenyl D-glucopyranoside (*p*NP-α-Glc and *p*NP-β-Glc), 4-nitrophenyl 2-acetamido-2-deoxy-D-glucopyranoside (*p*NP-α-GlcNAc and *p*NP-β-GlcNAc), 4-nitrophenyl D-mannopyranoside (*p*NP-α-Man and *p*NP-β-Man), and 4-nitrophenyl D-xylopyranoside (*p*NP-α-Xyl and *p*NP-β-Xyl) were from Sigma–Aldrich, USA. 4-Formylphenyl β-D-allopyranoside (*p*FP-β-All) was from Tokyo Chemical Industry (TCI), Japan. 4-nitrophenyl 2-acetamido-2-deoxy-4-thio-β-D-glucopyranoside (*p*NP-4S-β-GlcNAc) was synthesized as described earlier.[36] Labeled acceptor substrate FCHASE-aminophenyl-β-GlcNAc was prepared according to ref. [37]. β-Galactosidase from *Streptococcus pneumoniae* was from Prozyme, USA. Sep-Pak C18 cartridges were from Waters, USA. All reagents were from Sigma–Aldrich. NMR spectra were obtained at 500 MHz on an INOVA Unity spectrometer (Varian, USA). ESI mass spectra were recorded using a API-3000 and a 4000 Q-trap mass spectrometer (Applied Biosystems, USA) Capillary electrophoresis (CE) was done using a P/ACE system 5510 and a P/ACE MDQ apparatus from Beckman Coulter, USA, equipped with a variable UV detector set to 260 nm. The runs were performed with bare fused silica capillaries.

Enzyme assay for recombinant β-1,4-galactosyltransferases: An end-point method was used for determining the activity of human β4GalT-1 as well as HP0826 and LgtB. Crude or purified enzyme was mixed with 50 mM HEPES pH 7.5, 1 mm $MnCl_2$, 1.5 mM *p*NP-β-GlcNAc, and 1.5 mM UDP-Gal (all final concentrations). The activity assay was incubated at 37 °C and samples (20 μL) were taken at various time points. The incubation was stopped by heating samples to 95 °C for 5 min and centrifuging for 15 min at 13000 rpm. To the supernatant 20 μL H_2O and 10 μL 100 mM EDTA were added. Samples were analyzed by CE. An unmodified fused-silica capillary (I.D. 50 μm, 60 cm total capillary length, with a detection window at 47 cm) was used; separations were run at + 25 kV

(53 μA) and 25 °C using borate/SDS-buffer (20 mM $Na_2B_4O_7$ pH 9.4, 50 mM SDS). Samples were injected by pressure (10 s at 0.5 psi in forward direction) and separated within 20 min migration time. Capillaries were regenerated with a 2 min wash with each of: 0.2 M NaOH, water, running buffer. Quantification of analysis results was done using 32 Karat Software (Beckmann-Coulter, USA).

Construction and transformation of expression vector pHP21 for HP0826: HP0826 was cloned as the full-length version according to ref. [7]. PCR was performed to introduce restriction sites NdeI and SalI using primers 5′-GGGGGGCATATGCGTGTTTTTGCCATTTCTTTAAATC-3′ and 5′-CATACAAACTGCCAATATTTCAAATATTTAAAATGG-3′. Ligation into NdeI and SalI digested vector pCWori+[38] gave expression vector pHP21. After transformation, ompT protease deficient *E. coli* strain AD202 (F^- *ΔaraD*139 DE(*argF-lac*)169 *ompT*1000::*kan* LAM^- *flhD*5301 *fruA*25 *relA*1 *rpsL*150(str^R) *rbsR*22 *deoC*1)[39] was used for expression.

Production of HP0826, LgtB, and HB4GT: The LgtB full-length construct used was described in ref. [1]. Human β4GalT-1-maltose-binding protein fusion (HB4GT) was cloned as published.[40] LgtB, HB4GT, and HP0826 were expressed in *E. coli* AD202. For enzyme production 20 mL-cultures of 2X YT microbial medium containing 150 μg mL^{-1} ampicillin were inoculated and grown at 37 °C and 200 rpm overnight. 200 mL cultures containing the same ampicillin concentration were inoculated from the 20 mL starter culture to an OD_{600} of 0.25 and incubated at 25 °C and 200 rpm. Once an OD_{600} of 0.4 was reached cultures were induced with 0.5 mM IPTG and cells were grown for 24 h. By centrifuging with a Sorvall SLA-3000 rotor (Thermo Fisher Scientific, USA) at 5000 rpm for 30 min. cells were collected and stored at −20 °C until further use.

Purification of HP0826: A 10% (*w*/*w*) cell suspension was prepared in buffer A (100 mM HEPES/NaOH pH 8.0) and disrupted by high pressure in an Emulsiflex C-5 (Avestin, Canada). After centrifugation at 15000 rpm in a SS-34 rotor (Thermo Fisher Scientific, USA) the supernatant (40 mL) was collected and used for purification on a 20 mL DEAE-Sepharose Fast Flow (weak anion exchange; GE Healthcare, USA) column at a flow-rate of 5 mL min^{-1}. The column had been equilibrated with buffer A. HP0826 containing flow-through fractions were collected and pooled, proteins binding to the column were eluted with buffer B (buffer A + 1 M NaCl). HP0826 activity was determined as described above. SDS-PAGE analysis was performed according to ref. [41].

Purification of LgtB: A 10% (*w*/*w*) cell suspension was prepared in buffer A (100 mM HEPES/NaOH pH 8.0) and disrupted by high pressure in an Emulsiflex C-5 (Avestin, Canada). After centrifugation at 15000 rpm in a Sorvall SS-34 rotor (Thermo Fisher Scientific, USA) the supernatant (15 mL) was collected and used for purification on a 20 mL Q-Sepharose Fast Flow (strong anion exchange; GE Healthcare, USA) column at a flow rate of 5 mL min^{-1}. The column had been equilibrated with buffer A. LgtB containing flow-through fractions were collected and pooled, proteins binding to the column were eluted with buffer B (buffer A + 1 M NaCl). LgtB activity was determined as described above.

Purification of HB4GT: A 10% (*w*/*w*) cell suspension was prepared in buffer A (50 mM ammonium acetate pH 7.5, 10% glycerol) and disrupted by high pressure in an Emulsiflex C-5 (Avestin, Canada). After centrifugation at 15000 rpm in a SS-34 rotor (Thermo Fisher Scientific, USA) the supernatant (5 mL) was collected, diluted 1:1 with buffer A, and used for purification on a 8 mL Amylose Resin High Flow (New England Biolabs, USA) column at a flow rate of 5 mL min^{-1}. The column had been equilibrated with buffer A. Elution was done with buffer B (buffer A + 20 mM maltose). HB4GT containing elution fractions were collected and pooled. HB4GT activity was determined as described above.

Determination of biochemical data for HP0826: GalT activity was determined as described above with the following modifications. In order to examine the pH optimum of HP0826 we used HEPES buffer (50 mM) in a pH range of 6.8–8.2 in 0.2 pH unit steps. For the examination of metal ion dependency the following metals were used in form of 1 mM $MeCl_2$: Ca, Co, Mg, and Mn. For a control reaction without metal ion influence 10 mM EDTA was added to the assay. For donor specificity assays 20 mM UDP-GalNAc, UDP-Glc, or UDP-GlcNAc, 1.5 mM labeled acceptor (FCHASE-aminophenyl-β-GlcNAc) and 5 mM $MnCl_2$ were used. Samples were taken at various time points and analyzed according to ref. [42].

Kinetic characterization of HP0826 with acceptor *p*NP-β-GlcNAc: The kinetic constants for acceptor substrate *p*NP-β-GlcNAc and donor substrate UDP-Gal were derived from initial rate analysis by variation of the concentration of one of the substrates. *p*NP-β-GlcNAc concentrations examined were 0 to 3.3 mM with a constant UDP-Gal value of 10 mM. UDP-Gal concentrations examined were 0 to 50 mM with a constant *p*NP-β-GlcNAc value of 1.5 mM. Assays and activity determinations were performed as described above. Kinetic constants were calculated according to the Michaelis–Menten equation by use of the software Prism (Graph Pad Software, USA).

Acceptor substrate screening for HP0826: The α and β anomers of *p*NP-Gal, *p*NP-GalNAc, *p*NP-Glc, *p*NP-GlcNAc, *p*NP-Man, and *p*NP-Xyl as well as *p*FP-β-All and *p*NP-4S-β-GlcNAc were tested as acceptor substrates for HP0826. 20 mM UDP-Gal, 1.5 mM acceptor and 5 mM $MnCl_2$ (10 mM $MnCl_2$ for *p*NP-4S-β-GlcNAc, also 10 mM DTT to prevent disulfide formation) were used and the activities determined as described above. For *p*NP-Glc and *p*NP-4S-β-GlcNAc the conditions for CE analysis had to be modified by using + 15 kV (28 μA) and simple borate buffer (25 mM $Na_2B_4O_7$ pH 9.4), all other parameters remaining unchanged.

Acceptor substrate examinations for LgtB and HB4GT: HB4GT and LgtB were tested for activity with *p*NP-β-Man and *p*NP-4S-β-GlcNAc. For HB4GT 1.5 mM UDP-Gal, 1.5 mM of the acceptors and 1 mM $MnCl_2$ were used. For LgtB we utilized 20 mM UDP-Gal, 1.5 mM of the acceptors, and 1 mM $MnCl_2$. Activities were determined as described above.

Yield–time curve for HP0826 transfer of UDP-Gal to *p*NP-4S-β-GlcNAc: For this experiment 20 mM UDP-Gal, 1.5 mM *p*NP-4S-β-GlcNAc, 10 mM $MnCl_2$, 50 mM HEPES pH 7.2, and 75 mU of HP0826 were used in a volume of 250 μL and incubated at 37 °C. Time points were taken after 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 7 h, and 9 h. Samples were stopped and analyzed by CE as described above.

Synthesis of Gal-β-S-1,4-GlcNAc-β-*p*NP (5) with HP0826: For synthesis on the 0.5 mg scale the reactions contained: 20 mM UDP-Gal, 1.5 mM *p*NP-4S-β-GlcNAc, 10 mM $MnCl_2$, 50 mM HEPES pH 7.2, and 150 mU of HP0826 in a volume of 500 μL and incubated for 5 h at 37 °C. Quantitative conversion was monitored by CE as described above. The synthesis was stopped by heating to 95 °C for 5 min and centrifuging for 15 min at 13000 rpm. The supernatant was diluted 1:2 with H_2O and purified via a Sep-Pak C18 cartridge. The cartridge had been equilibrated with 10 mL acetonitrile and 10 mL H_2O. After application of the sample the cartridge was washed with 8 mL H_2O and eluted with 1 mL-steps of 10–30% and 100% acetonitrile. Product containing elution fraction (30% aceto-

nitrile) was dried in a SpeedVac (without heat). 0.35 mg (88% yield) were isolated.

Acceptor concentration variation for *p*NP-4S-β-GlcNAc with HP0826: *p*NP-4S-β-GlcNAc concentrations examined were 0 to 2.4 mM with a constant UDP-Gal value of 20 mM. HP0826 was used in a concentration of 300 mU mL^{-1}. Assays and activity determinations were performed as described above.

Galactosidase treatment of Gal-β-S-1,4-GlcNAc-β-*p*NP (5): The β-galactosidase from *Streptococcus pneumoniae* specific for Gal β1,4-cleavage[43] was used for this experiment. 1.5 mM of Gal-β-S-1,4-GlcNAc-β-*p*NP and 50 mU mL^{-1} galactosidase in reaction buffer (Prozyme, USA, total volume 50 μL) were incubated for 22 h. After the sample had been heated to 95 °C for 5 min and centrifuged for 15 min at 13000 rpm in a microcentrifuge, it was analyzed by CE as described above. For a control reaction Gal-β-1,4-GlcNAc-β-*p*NP was used which was completely cleaved within 30 min.

Yield–time curve for HP0826 transfer of UDP-Gal to *p*NP-β-Man: For this experiment 20 mM UDP-Gal, 1.5 mM *p*NP-β-Man, 1 mM $MnCl_2$, 50 mM HEPES pH 7.2, and 400 mU (1.2 mg) of HP0826 were used in a volume of 300 μL and incubated at 37 °C. Time points were taken after 10 min, 30 min, 1 h, 2 h, 4 h 45 min, 6 h 15 min, 8 h, 22 h 30 min, 30 h, and 40 h. Samples were stopped and analyzed by CE as described above.

Yield–time curve for LgtB transfer of UDP-Gal to *p*NP-β-Man: 20 mM UDP-Gal, 1.5 mM *p*NP-β-Man, 1 mM $MnCl_2$, 50 mM HEPES pH 7.2, and 3.1 mU (1.2 mg) of LgtB were used in a volume of 300 μL and incubated at 37 °C. Time points were taken after 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 26 h 30 min, 30 h, and 40 h. Samples were stopped and analyzed by CE as described above.

Synthesis of Gal-β-1,4-Man-β-*p*NP (6) with HP0826: For synthesis on the 1.5 mg scale 20 mM UDP-Gal, 1.5 mM *p*NP-β-Man, 1 mM $MnCl_2$, 50 mM HEPES pH 7.2, and 500 mU of HP0826 were used in a volume of 830 μL and incubated for 30 h at 37 °C. Quantitative conversion was monitored by CE as described above. The synthesis was stopped by heating to 95 °C for 5 min and centrifuging for 15 min at 13000 rpm. The supernatant was diluted 1:2 with H_2O and purified via a Sep-Pak C18 cartridge as described above. The product containing elution fractions (20 and 30% acetonitrile) were dried in a SpeedVac (without heat). 1.38 mg (90% yield) were isolated.

Galactosidase treatment of Gal-Man-β-*p*NP product synthesized with LgtB: The β-galactosidase from *Streptococcus pneumoniae* which is specific for Gal β1,4-cleavage[43] was used for this experiment. 1.5 mM of Gal-β-1,4-Man-β-*p*NP and 50 mU mL^{-1} galactosidase in reaction buffer (Prozyme, USA, total volume 50 μL) were incubated for 20 h. After heating the sample to 95 °C for 5 min and centrifuging for 15 min at 13000 rpm it was analyzed by CE as described above.

Acceptor concentration variation for *p*NP-β-Man with HP0826: *p*NP-β-Man concentrations examined were 0 to 36 mM with a constant UDP-Gal value of 20 mM. HP0826 was used in a concentration of 690 mU mL^{-1}. Assays and activity determinations were performed as described above.

Kinetic characterization of LgtB with acceptor *p*NP-*β*-Man: The kinetic constants for *p*NP-β-Man were derived from initial rate analysis by variation of the acceptor substrate with a constant UDP-Gal value of 20 mM. *p*NP-β-Man concentrations examined were 0 to 36 mM. Assays and activity determinations were performed as described above. Kinetic constants were calculated according to the Michaelis–Menten equation by use of the software Prism (Graph Pad Software, USA).

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