Highly efficient chemoenzymatic synthesis of β1–3-linked galactosides†

Hai Yu,[a] Vireak Thon,[a] Kam Lau,[a] Li Cai,[b] Yi Chen,[a] Shengmao Mu,[a] Yanhong Li,[a] Peng George Wang[b] and Xi Chen*[a]

Received 28th July 2010, Accepted 16th August 2010
DOI: 10.1039/c0cc02850a

A novel D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase cloned from *Bifidobacterium infantis* (BiGalHexNAcP) was used with a recombinant *E. coli* K-12 galactokinase (GalK) for efficient one-pot two-enzyme synthesis of T-antigens, galacto-*N*-biose (Galβ1–3GalNAc), lacto-*N*-biose (Galβ1–3GlcNAc), and their derivatives.

β1–3-Linked galactosides such as galacto-*N*-biose (GNB, Galβ1–3**Gal**NAc) and lacto-*N*-biose (LNB, Galβ1–3**Glc**NAc) are key carbohydrate structures in nature. They are themselves, or are part of, important carbohydrate epitopes involved in cell adhesion, signalling, fertilization, differentiation, development, and cancer metastasis. For example, GNB with an α-configuration at the anomeric carbon of GalNAc (Galβ1–3GalNAcα) is named Thomsen–Friedenreich antigen (TF or T-antigen, Galβ1–3GalNAcαSer/Thr) disaccharide. It is the glycan of the Core 1 mucin-type *O*-GalNAc glycopeptides/glycoproteins and can be branched/extended to form Core 2 as well as extended Core 1 and Core 2 glycans.[1] On the other hand, GNB with a β-configuration at the anomeric carbon of GalNAc (Galβ1–3GalNAcβ) is an essential part of the carbohydrate moieties of complex glycosphingolipids such as GA1, GM1, GD1, GT1, GQ1, and GP1, *etc*. In comparison, LNB is a common structure presented broadly in human milk oligosaccharides (*e.g*. lacto-*N*-tetraose and its sialylated/fucosylated derivatives) and in the carbohydrate moieties (*e.g*. type I glycans, Lewis a, sialyl Lewis a, and Lewis b antigens) of glycoproteins and glycolipids.[1,2]

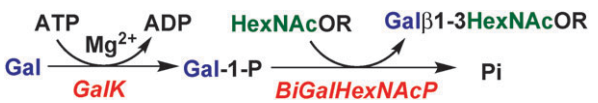
To use these β1–3-linked galactosides as probes or glycan building blocks for biological studies, GNB and LNB have been synthesized by chemical[3] and enzymatic approaches using β-glycosidases[4] or β1–3-galactosyltransferases.[5] Nevertheless, these methods have limitations. Chemical synthesis requires multiple tedious protection–deprotection and purification procedures. Glycosidase-catalyzed reactions usually result in low yields and poor regioselectivity. The formation of β1–3-linked galactosidic bonds using β1–3-galactosyltransferases has been proven efficient, but its application is hampered by limited access to a sufficient amount of highly efficient glycosyltransferases and the required sugar nucleotide, uridine 5′-diphosphate galactose (UDP-Gal).

[a] *Department of Chemistry, University of California, One Shields Avenue, Davis, California 95616, USA. E-mail: chen@chem.ucdavis.edu; Fax: +1 530-752-8995; Tel: +1 530-754-6037*
[b] *Departments of Biochemistry and Chemistry, Ohio State University, Columbus, OH 43210, USA*

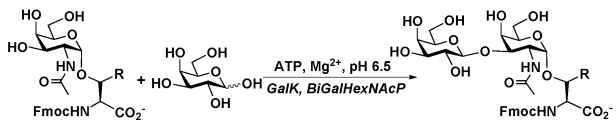
† Electronic supplementary information (ESI) available: Experimental details for cloning and characterization of BiGalHexNAcP, chemical and enzymatic synthesis, NMR and HRMS data. See DOI: 10.1039/c0cc02850a

Carbohydrate phosphorylases catalyze the reversible formation of monosaccharide-1-phosphate from an oligosaccharide or a polysaccharide and an inorganic phosphate. Their ability in catalyzing the reverse reaction has been used for the synthesis of oligosaccharides. Based on their protein sequence similarity, carbohydrate phosphorylases have been grouped into several families of glycoside hydrolases (GH13, GH65, GH94, GH112) and glycosyltransferases (GT3, GT4, GT20, GT35) in the Carbohydrate Active Enzymes (CAZy) database (http://www.cazy.org).[6] Among them, D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase (GalHexNAcP, EC 2.4.1.211), a member of CAZy glycoside hydrolase GH112 family, was initially reported in *Bifidobacterium bifidum* DSM 20082 in 1999.[7] It reversibly phosphorolyzes GNB and LNB to produce α-D-galactose-1-phosphate (Gal-1-P) and the corresponding *N*-acetyl-D-hexosamine. The partially purified enzyme (MW: 140 kDa)[7] was used in the synthesis of Galβ1–3GlcNAcαOR containing different aglycones with low yields.[8] The GalHexNAcP gene was later found in *Clostridium perfringens* ATCC13124, *Vibrio vulnificus* CMCP6, and several strains of bifidobacteria and was believed to contribute to the intestinal colonization of the bacteria.[9] The enzyme has been cloned and used with a sucrose phosphorylase, a UDP-glucose-hexose-1-phosphate uridylyltransferase, and a UDP-glucose 4-epimerase for one-pot four-enzyme high-yield large-scale production of LNB or GNB from sucrose and GlcNAc or GalNAc in the presence of UDP-glucose and phosphate.[10]

Crystal structure of a recombinant GalHexNAcP from *Bifidobacterium longum* JCM1217 reveals a partially broken TIM barrel fold resembling a thermophilic β-galactosidase.[11] Nevertheless, the acceptor substrate specificity of GalHexNAcP has not been studied in detail and its application in efficient synthesis of GNB and LNB derivatives has not been fully

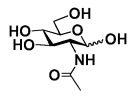
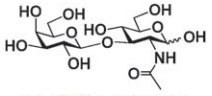
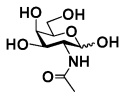
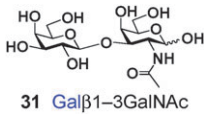
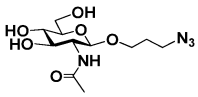
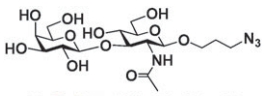
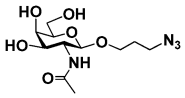
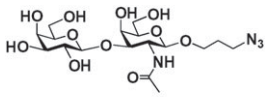
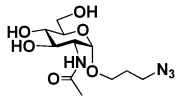
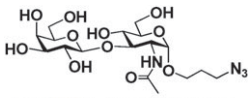
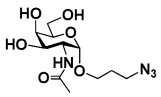
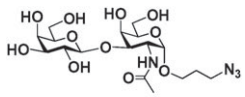
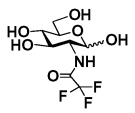
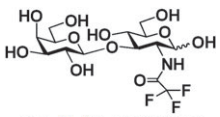
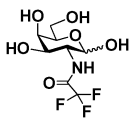
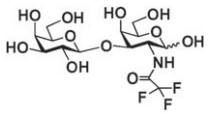
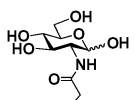
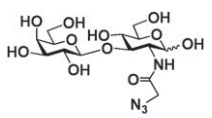
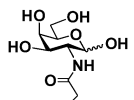
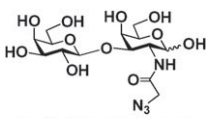
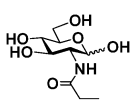
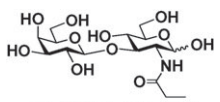
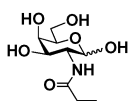
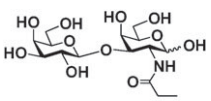
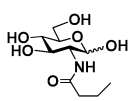
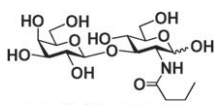
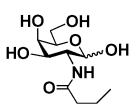
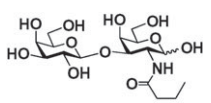
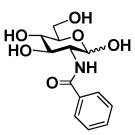
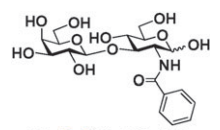
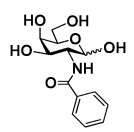
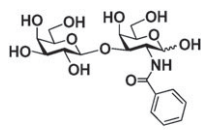
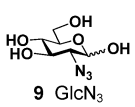
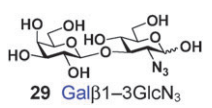
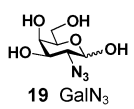
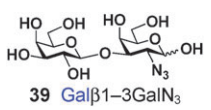
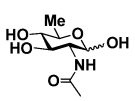
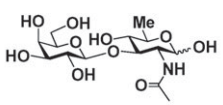
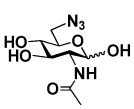
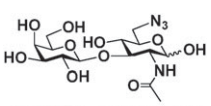


Scheme 1 Synthetic scheme for one-pot two-enzyme synthesis of β1–3-linked galactosides. GalK, *E. coli* K-12 galactokinase; BiGalHexNAcP, *Bifidobacterium infantis* D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase.



Scheme 2 One-pot two-enzyme synthesis of T-antigen Galβ1–3GalNAcα1-*O*-Ser/Thr.

Table 1 One-pot two-enzyme synthesis of β1–3-linked galactosides from Gal, ATP, and *N*-acetyl-hexosamine (HexNAc) derivatives using GalK and BiGalHexNAcP

Acceptors	Products	Yields (%)	Acceptors	Products	Yields (%)
 **1** GlcNAc	 **21** Galβ1–3GlcNAc	95	 **11** GalNAc	 **31** Galβ1–3GalNAc	93
 **2** GlcNAcβProN$_3$	 **22** Galβ1–3GlcNAcβProN$_3$	96	 **12** GalNAcβProN$_3$	 **32** Galβ1–3GaNAcβProN$_3$	95
 **3** GlcNAcαProN$_3$	 **23** Galβ1–3GlcNAcαProN$_3$	94	 **13** GalNAcαProN$_3$	 **33** Galβ1–3GalNAcαProN$_3$	92
 **4** GlcNTFA	 **24** Galβ1–3GlcNTFA	93	 **14** GalNTFA	 **34** Galβ1–3GalNTFA	94
 **5** GlcNAcN$_3$	 **25** Galβ1–3GlcNAcN$_3$	91	 **15** GalNAcN$_3$	 **35** Galβ1–3GalNAcN$_3$	92
 **6** GlcNPr	 **26** Galβ1–3GlcNPr	86	 **16** GalNPr	 **36** Galβ1–3GalNPr	69
 **7** GlcNBu	 **27** Galβ1–3GlcNBu	78	 **17** GalNBu	 **37** Galβ1–3GalNBu	NR
 **8** GalNBz	 **28** Galβ1–3GalNBz	NR	 **18** GalNBz	 **38** Galβ1–3GalNzBz	NR
 **9** GlcN$_3$	 **29** Galβ1–3GlcN$_3$	NR	 **19** GalN$_3$	 **39** Galβ1–3GalN$_3$	NR
 **10** GlcNAc6Deoxy	 **30** Galβ1–3GlcNAc6Deoxy	84	 **20** GlcNAc6N$_3$	 **40** Galβ1–3GlcNAc6N$_3$	87

explored. Here, we report a highly efficient one-pot two-enzyme approach for the synthesis of diverse β1–3-linked galactosides, including T-antigens, GNB, LNB, and their derivatives using a novel D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase cloned from *Bifidobacterium infantis* (BiGalHexNAcP) and a recombinant galactokinase (GalK) cloned from *E. coli* K-12.[12]

To obtain BiGalHexNAcP, *Blon_2174* gene (GenBank NC_011593) was amplified from the genomic DNA of *Bifidobacterium longum* subsp. *infantis* ATCC 15697[13] by polymerase chain reaction and cloned into pET15b vector. Optimal expression of the recombinant N-His$_6$-tagged protein (MW: 86.5 kDa) was achieved by incubating *E. coli* Origami™ B(DE3) cells containing desired plasmids at 25 °C for 18–20 h with vigorous shaking (250 rpm) after the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.1 mM). Ni^{2+}–NTA column purification using an AKTA FPLC system yielded 55 mg pure protein per litre of cell culture.

As shown in Scheme 1, the synthesis of GNB, LNB, and their derivatives was carried out using a one-pot two-enzyme system containing BiGalHexNAcP and a recombinant *E. coli* GalK. GalK catalyzed the formation of α-D-Gal-1-P from inexpensive D-Gal in the presence of ATP. The *in situ* generated α-D-Gal-1-P was used as the donor substrate by the phosphorylase to form diverse β1–3-linked galactosides. Excess amounts of D-Gal and ATP (1.2 or 1.5 equiv.) were used to drive the reaction towards disaccharide formation.

HPLC-based pH profile study of BiGalHexNAcP revealed that its catalytic activity was optimum in a relatively narrow pH range of 5.0 to 6.5. Extremely low activity was observed when the pH of the reaction reached 7.0 or higher (see ESI†). For GalK, it prefered a pH higher than 7.0 for optimum activity, showed medium activity at pH 6.5 and low activity at pH lower than 6.5. Therefore, pH 6.5 was chosen for the one-pot two-enzyme reactions. The reactions were carried out at 37 °C for 48 h. Products were purified using the combination of size exclusion chromatography and silica gel chromatography.

As shown in Table 1, BiGalHexNAcP exhibits promiscuous acceptor substrate specificity and has comparable levels of activity toward GlcNAc and GalNAc-based structures. For example, free GlcNAc (**1**) and GalNAc (**11**), their β-glycosides (**2** and **12**, respectively) and α-glycosides (**3** and **13**, respectively) are superb acceptors for BiGalHexNAcP to produce LNB (**21–23**) and GNB (**31–33**) disaccharides in excellent 92–96% yields. Apparently, the configurations of the C-4 hydroxyl group and the anomeric center of the *N*-acetyl hexosamine do not affect the activity of BiGalHexNAcP.

Similarly, *N*-acetyl group derivatization with small acyl groups or derivatives does not affect the BiGalHexNAcP activity significantly. For example, GlcNTFA **4** and GalNTFA **14** with an *N*-trifluoroacetyl group or $GlcNAcN_3$ **5** and $GalNAcN_3$ **15** with an *N*-azidoacetyl group in the hexosamines are excellent acceptors to produce disaccharides **24–25** and **34–35** in 91–94% yields. However, larger *N*-acyl groups differentiated glucosamine and galactosamine-based acceptors. For example, GlcNPr **6** and GlcNBu **7** with an *N*-propyl and an *N*-butyl group, respectively, at glucosamine, are good acceptors for BiGalHexNAcP to synthesize disaccharides **26** and **27** in 86% and 78% yields, respectively. The synthetic yield decreases moderately as the size of the *N*-acyl group on the glucosamine increases. In contrast, the corresponding galactosamine derivative GalNPr **16** leads to disaccharide **36** in a moderate 69% yield while GalNBu **17** is not a suitable acceptor for BiGalHexNAcP. A bulky *N*-benzoyl group prevents both glucosamine and galactosamine derivatives (**8** and **18**) from being suitable BiGalHexNAcP acceptors. Quite interestingly, $GlcN_3$ **9** and $GalN_3$ **19**, the C2 derivatives of GlcNAc and GalNAc with the *N*-acetyl group being replaced by a relatively small azido group ($-N_3$), are not tolerable acceptors.

BiGalHexNAcP also shows good tolerance towards C6-modification. Both 6-deoxy-GlcNAc (GlcNAc6Deoxy) **10** and 6-azido-6-deoxy-GlcNAc ($GlcNAc6N_3$) **20** are very good acceptors for the enzyme to synthesize disaccharides **30** and **40** in high yields (84 and 87%, respectively).

The BiGalHexNAcP and the one-pot two-enzyme system have also been successfully applied in the efficient synthesis of biologically important T-antigens Galβ1–3GalNAcα1-*O*-Ser and Galβ1–3GalNAcα1-*O*-Thr. As shown in Scheme 2, incubating GalNAcα1-*O*-Ser **41** or GalNAcα1-*O*-Thr **42** with Gal and ATP in the presence of GalK and BiGalHexNAcP successfully produced the desired disaccharide products **43** and **44** in excellent 92% and 91% yields, respectively.

In summary, taking advantage of the acceptor substrate promiscuity of BiGalHexNAcP, we have developed a highly efficient one-pot two-enzyme approach for the synthesis of diverse β1–3-linked galactosides. Compared to galactosyltransferase-catalyzed approaches, the BiGalHexNAcP-catalyzed reactions do not require the use, *in situ* generation, or regeneration of expensive sugar nucleotides, and thus are more efficient and simplified systems for producing galactosides. We believe that this synthetic route will contribute greatly to obtaining and elucidating the important roles of β1–3-galactosides as well as β1–3-galactoside-containing glycans and glycoconjugates.

This work was supported by NIH grants R01GM076360, U01CA128442 (to X. Chen), and R01HD061935 (to P. G. Wang). X. Chen is an Alfred P. Sloan Research Fellow, a Camille Dreyfus Teacher-Scholar, and a UC-Davis Chancellor's Fellow. We thank Professor David Mills at the University of California-Davis for providing us the genomic DNA of *Bifidobacterium longum* subsp. *infantis* ATCC 15697.

Notes and references

1 *Essentials of Glycobiology*, ed. A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart and M. E. Etzler, Cold Spring Harbor Laboratory Press, New York, 2nd edn, 2008.
2 (*a*) C. Kunz, S. Rudloff, W. Baier, N. Klein and S. Strobel, *Annu. Rev. Nutr.*, 2000, **20**, 699–722; (*b*) J. Holgersson and J. Lofling, *Glycobiology*, 2006, **16**, 584–593; (*c*) T. Feizi, *Immunol. Rev.*, 2000, **173**, 79–88; (*d*) C. R. Bertozzi and L. L. Kiessling, *Science*, 2001, **291**, 2357–2364.
3 M. Wilstermann and G. Magnusson, *Carbohydr. Res.*, 1995, **272**, 1–7.
4 (*a*) L. Hedbys, E. Johansson, K. Mosbach and P. O. Larsson, *Carbohydr. Res.*, 1989, **186**, 217–223; (*b*) A. Vetere, M. Miletich, M. Bosco and S. Paoletti, *Eur. J. Biochem.*, 2000, **267**, 942–949; (*c*) A. D'Almeida, M. Ionata, V. Tran, C. Tellier, M. Dion and C. Rabiller, *Tetrahedron: Asymmetry*, 2009, **20**, 1243–1246.
5 (*a*) G. Baisch, R. Ohrlein, M. Streiff and F. Kolbinger, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 751–754; (*b*) D. M. Su, H. Eguchi, W. Yi, L. Li, P. G. Wang and C. Xia, *Org. Lett.*, 2008, **10**, 1009–1012; (*c*) Z. Wang, M. Gilbert, H. Eguchi, H. Yu, J. Cheng, S. Muthana, L. Zhou, P. G. Wang, X. Chen and X. Huang, *Adv. Synth. Catal.*, 2008, **350**, 1717–1728.
6 B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard and B. Henrissat, *Nucleic Acids Res.*, 2009, **37**, D233–D238.
7 D. Derensy-Dron, F. Krzewinski, C. Brassart and S. Bouquelet, *Biotechnol. Appl. Biochem.*, 1999, **29**(Pt 1), 3–10.
8 E. Farkas, J. Thiem, F. Krzewinski and S. Bouquelet, *Synlett*, 2000, 728–730.
9 (*a*) M. Kitaoka, J. Tian and M. Nishimoto, *Appl. Environ. Microbiol.*, 2005, **71**, 3158–3162; (*b*) J. Xiao, S. Takahashi, M. Nishimoto, T. Odamaki, T. Yaeshima, K. Iwatsuki and M. Kitaoka, *Appl. Environ. Microbiol.*, 2010, **76**, 54–59.
10 (*a*) M. Nishimoto and M. Kitaoka, *Biosci., Biotechnol., Biochem.*, 2007, **71**, 2101–2104; (*b*) M. Nishimoto and M. Kitaoka, *Carbohydr. Res.*, 2009, **344**, 2573–2576.
11 M. Hidaka, M. Nishimoto, M. Kitaoka, T. Wakagi, H. Shoun and S. Fushinobu, *J. Biol. Chem.*, 2009, **284**, 7273–7283.
12 X. Chen, J. Fang, J. Zhang, Z. Liu, J. Shao, P. Kowal, P. Andreana and P. G. Wang, *J. Am. Chem. Soc.*, 2001, **123**, 2081–2082.
13 D. A. Sela, J. Chapman, A. Adeuya, J. H. Kim, F. Chen, T. R. Whitehead, A. Lapidus, D. S. Rokhsar, C. B. Lebrilla, J. B. German, N. P. Price, P. M. Richardson and D. A. Mills, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 18964–18969.