Xylose: The First Ambident Acceptor Substrate for Galactosyltransferase from Bovine Milk

Torsten Wiemann,† Yoshihiro Nishida,‡ Volker Sinnwell, and Joachim Thiem*

Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany

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In the past few years the acceptor substrate specificity of β1,4-galactosyltransferase (GalT) from bovine milk has been investigated extensively by various groups. In each case, the β1,4-galactosylated products have been observed exclusively. Recently we reported on a frame-shifted recognition of modified acceptor substrates resulting in the formation of β,β-trehalose type disaccharides. In an extension to these investigations we have now found that xylose is recognized by the enzyme both in the normal and in the reverse orientation. Therefore galactosylation of xylose results in a mixture of β1,4- and β1,β1-galactopyranosyl xylopyranosides. Synthesis and preparation of both disaccharides and their behavior toward β-galactosidase from *E. coli* are described. The results lead to some implications about the active site of GalT.

Galactosyltransferase (GalT) is of central importance in lactose biosynthesis and glycoprotein assembly. Furthermore it is the most accessible of all glycosyltransferases. Therefore GalT has been the object of extensive research including studies on its substrate specificity[1-3] and its application in the chemoenzymatic synthesis of carbohydrates.[4-7] Apart from recently observed exceptions, each identified product proved to be a β1,4-galactosylated derivative. The exceptions discovered so far are 3-*N*-acetylamino sugars, namely *N*-acetylgentosamine,[8] *N*-acetylkanosamine,[9] and *N*-acetyl-5-thiogentosamine.[10] The galactosylation of 3-*N*-acetylamino sugars is highly regio- and stereospecific, but instead results in β1,β1-linked disaccharides. No byproducts could be detected. It may be assumed that the 3-*N*-acetylamino group is responsible for the frame-shifted recognition of the acceptor substrate.

Galactosylation of xylose does not seem to play a role in the metabolism of xylose.[11] However it is well-known that in the presence of α-lactalbumin, xylose is recognized by GalT as an acceptor substrate.[12] These investigations were performed on an analytical scale, applying a normal biochemical assay procedure. It is a fundamental drawback of such enzymatic assays that only the reaction turnover is measured which does not give any information on the structure of the products.

In the present study we have reexamined the galactosylation of xylose by galactosyltransferase from bovine milk on a preparative scale. The results, along with those earlier obtained,[8-10] are discussed with respect to structural features of the acceptor–substrate binding site.

The galactosylation was performed using the two-enzyme system depicted in Figure 1. UDP-glucose (**1**) is epimerized by UDP-galactose-4-epimerase to give UDP-galactose (**2**). By catalysis of the GalT/α-lactalbumin complex, UDP-galactose reacts with xylose (**3**) to give the product disaccharides and UDP as a byproduct. The reaction is worked up in the reported way.[7c] Performing the reaction on a 200 μmol scale and using 5 U of GalT, a yield of 50% was obtained. Since TLC showed one single product there was no indication of any side-reaction. Only when measuring the ^{1}H-NMR spectra it became obvious that a mixture of products had been obtained (cf. Figure 2a). On analysis and by comparison with NMR spectra of similar compounds, it became evident that xylose had been galactosylated not only in the 4-position but also in the β1-position. Thus the five doublets which appear in the anomeric region (cf. Figure 2) can be attributed to H-1 (α- and β-anomer, respectively) and H-1′ of the reducing disaccharide **4**, and H-1 and H-1′ of the nonreducing disaccharide **7**. The signal of H-1′ of **4** appears as a doublet of doublets owing to a slight difference in the chemical shifts of the α- and β-anomers.

Further evidence was obtained by NOE experiments (Figure 3). A strong NOE was observed on irradiation at H-1 and H-1′, respectively, of **7**, and was in the same order of magnitude as the NOE observed for the other axial protons H-3, H-5, H-3′, and H-5′ (5.4 to 7.8%, cf. Figure 3). Thus the cis-periplanar position of H-1 and H-1′ was confirmed. Irradiation at H-1 of **4** (β-anomer)

† Present address: The Scripps Research Institute, Department of Molecular Biology, MB 20, 10666 North Torrey Pines Road, La Jolla, California 92037.

‡ Present address: Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori Amamiyamachi, Aobaku, Sendai 980, Japan

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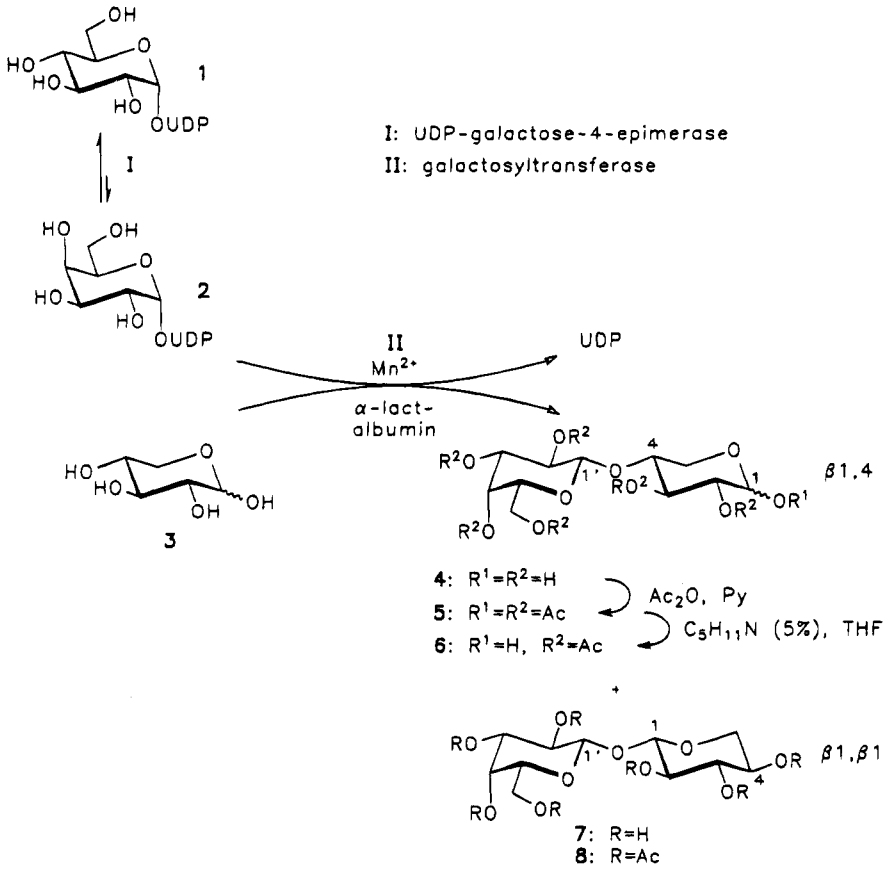

Figure 1. Galactosyltransferase-catalyzed galactosylation of xylose.

gives an NOE only at H-3 and H-5 and has no observable effect on any other doublets in the anomeric region.

At room temperature the anomeric protons of the nonreducing disaccharide **7** are partially overlapped by the HDO-signal (cf. Figure 2). Raising the temperature to 328 K caused a high-field shift of the HDO-signal allowing a proper integration of the H-1 and H-1′ signals. According to this experiment **4** and **7** are present in a ratio of 2:1.

These results may be rationalized by comparing the shapes of β-D-xylose in its normal and reverse orientation (Figure 4). Except for the positions of the ring oxygen and the isosteric 5-methylene group the normal and reverse orientation are essentially the same. From the enzyme's point of view merely an exchange of the ring oxygen and the methylene group has occurred but the exact ratio of the products might be a consequence of several factors:

(i) The oxygen/methylene switch might cause different binding constants of the enzyme–substrate and enzyme–product complexes.

(ii) Since galacto-configured sugars are not recognized as acceptor substrates[13], reverse orientated D-xylose will be fixed at the acceptor binding site only with the anomeric hydroxy group being in equatorial orientation, i.e. in its β-form. On the other hand galactosyltransferase accepts both the α- and β-form of the normal orientated sugar provided that α-lactalbumin is present and provided that the aglycon is small.[14] Therefore the apparent concentration of xylose for the formation of the

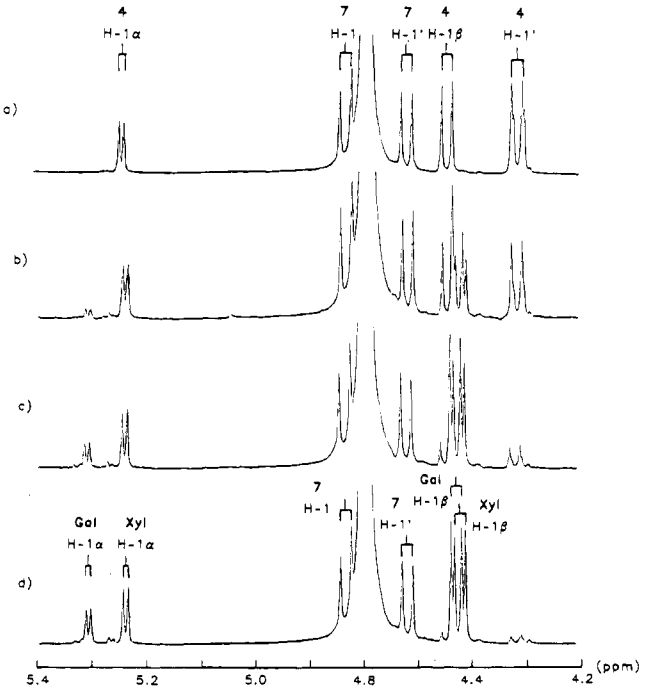

Figure 2. Anomeric region of starting disaccharides **4** and **7** and galactose (Gal) and xylose (Xyl) obtained by cleavage with β-galactosidase. (a) Anomeric region of **4** and **7** before adding β-galactosidase. (b) 4 h after adding β-galactosidase. (c) 7 h after adding β-galactosidase. (d) 10 h after adding β-galactosidase. The signals of compound **4** disappeared whereas those of Gal and Xyl appeared. H-1α of Xyl has the same chemical shift as H-1α of **4**. Apparently **7** is unaffected by β-galactosidase.

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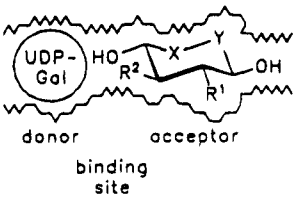
Table 1. Tabulation of Substituent Effects on Acceptor Ability[a]



		R^1	R^2	X	Y	substrate?	product
Xyl	n	OH	OH	CH_2	O	+	β1,4
	r	OH	OH	O	CH_2	+	β1,β1
Glc	n	OH	OH	e-$CH(CH_2OH)$	O	+	β1,4
	r	OH	OH	O	e-$CH(CH_2OH)$	−	
Glc3NAc	n	OH	NHAc	e-$CH(CH_2OH)$	O	−	
	r	NHAc	OH	O	e-$CH(CH_2OH)$	+	β1,β1
Xyl3NAc	n	OH	NHAc	CH_2	O	−	
	r	NHAc	OH	O	CH_2	+	β1,β1
GlcNAc	n	NHAc	OH	e-$CH(CH_2OH)$	O	+	β1,4
	r	OH	NHAc	O	e-$CH(CH_2OH)$	−	

[a] Abbreviations: Xyl, xylose; Glc, glucose; Glc3NAc, *N*-acetylkanosamine; Xyl3NAc, *N*-acetylgentosamine; GlcNAc, *N*-acetylglucosamine; n, normal orientation; r, reverse orientationl; e, equatorial).

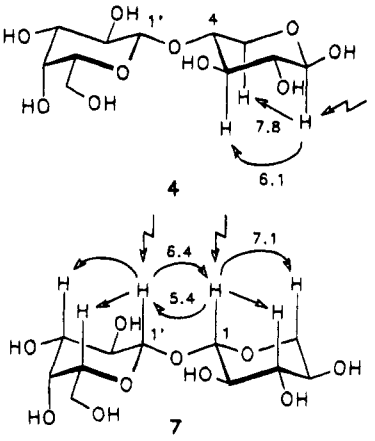


Figure 3. Observed nuclear Overhauser enhancements (NOE's) in **4** and **7**.

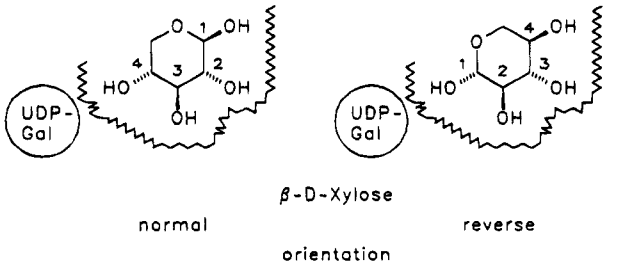


Figure 4. Normal and reverse orientation of β-D-xylopyranose.

β1,β1-disaccharide is probably much lower than for β1,4-galactosylation.

(iii) The nucleophilic reactivity of the anomeric hydroxy group is assumed to be more pronounced than that of the 4-OH group.

It is well known that GalT does not only recognize monosaccharides as acceptor substrates but also hetero-oligosaccharides having terminally β-linked glucose and *N*-acetylglucosamine units. In analogy we expected to find a second galactosylation of both **4** and **7** which would yield a nonreducing trisaccharide having the structure Gal-β1,4-Xyl-β1,β1-Gal. We were surprised not to find any indication of trisaccharide formation neither by gel permeation chromatography nor by NMR-measurements. Apparently the β-xylosides are poor substrates in the GalT-reaction.

Several attempts to separate **4** and **7** and even their peracetates **5** and **8** (cf. Figure 1) turned out to be unsuccessful under various conditions. Consequently, a chemical modification of **5** and **8** seemed to be the method of choice. Treatment of the mixture with piperidine in THF[15] (5% solution) overnight at room temperature resulted in a selective hydrolysis of the anomeric acetyl group of **5** (cf. Figure 1). Other acetyl groups are not affected. Separation of **6** and **8** was easily done and **5** could be regenerated by acetylation of **6**. The ^{1}H-NMR spectra of **5** and **8** confirmed the proposed structure.

In order to investigate the behavior of **4** and **7** towards β-galactosidase, the mixture of both compounds was incubated in D_2O with β-galactosidase from *Escherichia coli*. Reaction progress was monitored by ^{1}H-NMR. As depicted in Figure 2 the reducing disaccharide **4** is completely hydrolyzed in the course of several hours. The nonreducing disaccharide **7** does not seem to be affected and can be isolated by gel permeation chromatography. After peracetylation the ^{1}H-NMR spectra proved to be identical with that of compound **8**. Apparently the reducing disaccharide is a much better substrate for β-galactosidase.

The results known so far concerning the reverse orientation binding of acceptor substrates by the GalT are summarized in Table 1. Due to its high symmetry xylose is recognized in both the normal and the reverse orientation. Lowering the symmetry by an equatorial $CH(CH_2OH)$ group in the X or Y position (glucose, Glc) allows the formation of the enzyme–substrate complex only with the sugar in its normal orientation (X = CH-(CH_2OH), Y = O) since no β1,β1-galactosylation is being observed. Hence, an equatorial $CH(CH_2OH)$ group in the Y position is not favored. Nevertheless, this is not true with R^1 = NHAc, since *N*-acetylkanosamine (Glc3NAc) gives only the β1,β1-product (R^1 = NHAc, X = O, Y = $CH(CH_2OH)$).[9] This may be explained by assuming a positive contribution of an equatorial $CH(CH_2OH)$ group in Y position to the total binding energy which is offset by a large negative contribution of the NHAc group in R^1 and/or by assuming a change of the three-dimensional

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structure at the Y-binding site caused by the binding of the NHAc moiety in R^1. In either case it may be concluded that binding of the NHAc group at R^1 plays a major role in binding of *N*-acetylamino sugars. Lowering the symmetry of xylose by substitution of 3-OH by an NHAc group leads to *N*-acetylgentosamine (Xyl3NAc) which is recognized only in its reverse orientation (R^1 = NHAc, X = O, Y = CH_2).[8] Consequently an equatorial NHAc group at R^2 is not tolerated. This is surprising in so far as other bulky groups in this position are tolerated. In particular, *N*-acetylmuramic acid (R^2 = $CH_3CH(O)$-CO_2H) is a (moderate) acceptor substrate of GalT.[14] Apparently binding of the NHAc moiety in R^1 is much more favored as against binding in R^2, underlining the prominent role of this *N*-acetyl binding site.

In addition to a discussion of substituent effects already published[1] we may summarize the following:

(i) An equatorial $CH(CH_2OH)$ group at the Y position allows product formation only when an NHAc group at the R^1 position is present.

(ii) Binding of the NHAc group causes a large negative contribution to the total binding energy and/or a change of the three-dimensional structure of the enzyme.

(iii) The position of the NHAc moiety determines the formation of either β1,4- or β1,β1-galactosylated products.

The enzymatic galactosylation of xylose described here differs from other GalT-reactions in that xylose is galactosylated at two different positions. These findings give new insights into the binding site of GalT and may provide a valuable enzymatic access to some nonreducing oligosaccharides on a preparative scale.

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