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Galactosyl transfer ability of β-(1→4)-galactosyltransferase toward 5a-carba-sugars

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Abstract

Bovine β-(1→4)-galactosyltransferase was assayed with a series of 5a-carba-sugars, i.e., sugar analogues in which the ring oxygen of pyranose is replaced by a methylene group. The analogues are 5a-carba-sugar of 2-acetamido-2-deoxy-α-DL-galactopyranose, both α and β anomers of 2-acetamido-2-deoxy-DL-glucopyranose (5a-carba-DL-GlcNAc), and 2-acetamido-2-deoxy-DL-mannopyranose. Of these analogues, both α and β anomers of 5a-carba-DL-GlcNAc act as an acceptor. Enzymatic synthesis using the α and β anomers of 5a-carba-DL-GlcNAc afforded the corresponding D-Gal-β-(1→4)-5a-carba-α-D-GlcNAc and D-Gal-β-(1→4)-5a-carba-β-D-GlcNAc on a practical scale, and these structures were confirmed by NMR spectroscopy. These results indicate that the ring oxygen atom in the 5a-carba-D-GlcNAc is not used for specific recognition by bovine β-(1→4)-galactosyltransferase.

Keywords: Bovine β-(1→4)-galactosyltransferase; 5a-Carba-sugar; Enzymatic synthesis; Enzyme kinetics; Disaccharide

1. Introduction

Bovine β-(1→4)-galactosyltransferase (GalTase; EC 2.4.1.90) catalyses the transfer of the galactosyl residue from UDP-D-galactose to the 4-position of 2-acetamido-2-deoxy-D-glucose (GlcNAc). This enzyme is extensively studied in a series of glycosyltransferases. Enzymatic synthesis of galactosyloligosaccharides and investigation of the substrate specificity toward both UDP-D-galactose and GlcNAc residues have been performed by many research groups [1]. Investigation of the donor substrate specificity revealed that the enzyme specifically recognizes the 4- and 6-hydroxyl groups [2], and it further showed the possibility for modification at the 2-position with a large substituent such as a methoxy or acetamido group [3,4]. On the other hand, with respect to substrate specificity toward GlcNAc, the enzyme assay revealed that the acetamido group and the hydroxyl group at the 2- and 4-position, respectively, are essential functional groups for specific recognition by GalTase [5,6]. In addition, GalTase can bind GlcNAc derivatives having a large sub-

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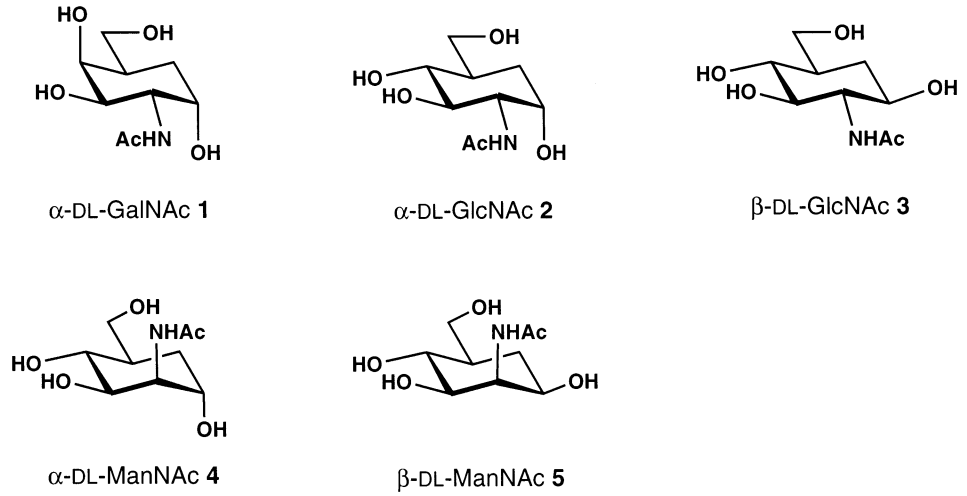


Fig. 1. Candidates for 2-acetamido-2-deoxy-5a-carba-hexopyranose acceptors. The formulae depict only the enantiomer corresponding to the 5a-carba-D-hexopyranoses.

stituent at the 6-position [6,7]. Consequently, under the conditions when both acceptor and donor bind, it could be deduced that the hydroxyl groups at the 2″-position of UDP-D-galactose and the 6-position of GlcNAc appear to be positioned outside of the combining site; thus a new tricomponent inhibitor was designed [8]. The inhibition assay with this compound showed very potent inhibitory activity toward GalTase. However, in the structure of GlcNAc, it has not been investigated whether GalTase recognizes the oxygen atom at the 5-position. Here we would like to report recognition by GalTase toward this oxygen atom.

2. Results and discussion

In order to investigate the recognition by GalTase toward the oxygen atom in the pyranoside ring, substitution of the oxygen for another atom or functional group is a convenient method. Sugar analogues having a nitrogen or sulfur atom instead of the oxygen atom at the 5-position have already been synthesized and assayed toward GalTase [9]. However, these are strictly glucose analogues and do not constitute a system for Gal-β-(1→4)-GlcNAc (LacNAc analogue) synthesis. In addition, since the nitrogen and sulfur atoms have lone pairs, the characteristics of the oxygen atom could not be fully removed by substitution with such atoms. Therefore, in order to investigate recognition by GalTase for the oxygen atom, we used a 5a-carba-sugar in which the 5-position is substituted by a methylene group. Use of this carba-sugar is advantageous in fixing the configuration of an anomeric hydroxyl group. We used five kinds of 5a-carba-sugars: 2-acetamido-2-deoxy-α-DL-galactopyranose **1** [10,11], 2-acetamido-2-deoxy-DL-glucopyranose (5a-carba-DL-GlcNAc) **2** (α), **3** (β) [10,11], and 2-acetamido-2-deoxy-DL-mannopyranose **4** (α), **5** (β) [10,11], as shown in Fig. 1.

In order to check the ability of these 5a-carba-sugars, we assayed to determine which 5a-carba-sugars could act as a galactosyl acceptor. As shown in Fig. 2, only 5a-carba-DL-

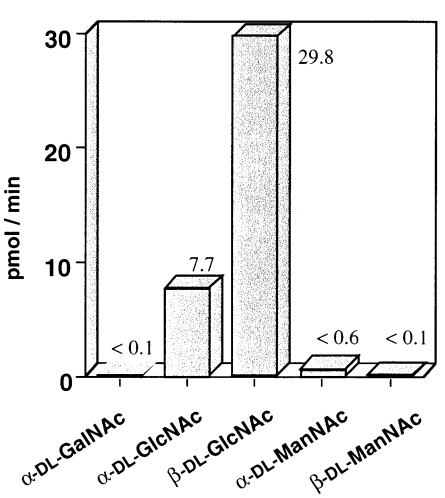
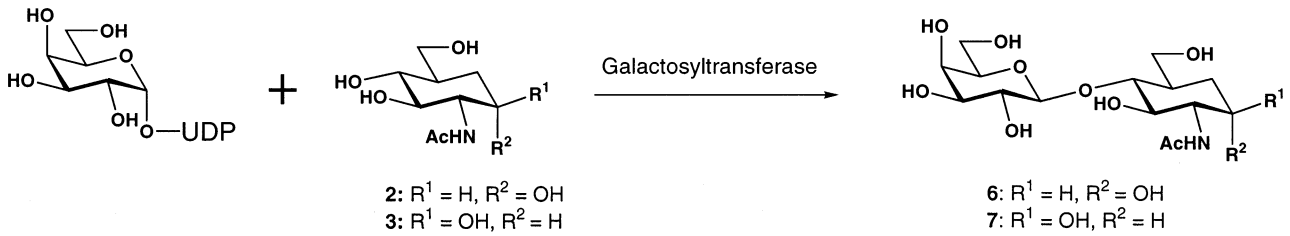


Fig. 2. Transfer assay toward 2-acetamido-2-deoxy-5a-carba-DL-hexopyranoses (20 mM).



Scheme 1. Galactosyl-transfer reaction.

Table 1
Kinetic parameters of 5a-carba-D-GlcNAc

	D-GlcNAc	5a-Carba-α-D-GlcNAc (**2**)	5a-Carba-β-D-GlcNAc (**3**)
K_m (mM)	1.56	19.74	2.52
V_{max} (pmol/min)	1.73	0.78	1.69
V_{max}/K_m	1.10	0.04	0.67

GlcNAc could accept galactosyl transfer. However, even though 5a-carba-DL-sugars could not act as an acceptor, they have the possibility of binding to GalTase. Therefore, we performed an inhibition assay with these 5a-carba-DL-sugars. However, when these 5a-carba-DL-sugars **1**, **4**, and **5** (20 mM) were evaluated as inhibitors toward an acceptor (GlcNAc: 3 mM), no inhibition activity was observed. These results indicate that the 5a-carba-DL-sugars **1**, **4**, and **5** do not bind to GalTase.

In order to confirm the products of the enzymatic assay, we performed practical enzymatic syntheses with 5a-carba-α-DL- **2** and 5a-carba-β-DL-GlcNAc **3**. The reactions afforded the disaccharides **6** and **7**, but half of each acceptor remained in each of the reactions. We concluded that 5a-carba-D-GlcNAc acted as a galactosyl acceptor, and its enantiomer remained unchanged. The newly formed glycosidic linkages were confirmed to be β-(1→4) by ^{1}H NMR and HMBC spectra (Scheme 1).

Kinetic parameters for 5a-carba-D-GlcNAc were also measured, and the results are summarized in Table 1. The K_m value of 5a-carba-β-D-GlcNAc is 1.6 times higher than that of native GlcNAc, and the V_{max} value is identical to that of GlcNAc. The K_m value of 5a-carba-α-D-GlcNAc is 12 times higher than that of GlcNAc, while the V_{max} value is about one-half of that of GlcNAc. Thus, 5a-carba-β-L-GlcNAc might not inhibit these galactosyl transfer reaction as the K_m value of 5a-carba-β-D-GlcNAc is close to that of natural GlcNAc.

Bock et al. reported the conformational properties of these 5a-carba-DL-GlcNAc derivatives [11]. Their NMR data suggested that the *gt* and *gg* rotamers are predominantly oriented around the 6-hydroxyl group. Since these conformational properties were in good agreement with that of the natural GlcNAc residue [12], substitution with a methylene group at the 5-position does not affect the conformational properties. Therefore, since the K_m value of 5a-carba-β-D-GlcNAc was slightly elevated over that of natural GlcNAc, GalTase appears to interact weakly with the oxygen atom at the 5-position.

Yuasa et al. reported that UDP-5″a-carba-α-D-galactose acts as an inhibitor toward GalTase [13]. Therefore, the oxygen in the pyranoside ring of both donor and acceptor is not involved in the specific recognition by GalTase. In addition, glucosaminyltransferase-V also does not recognize the oxygen atom at the 5-position of the mannosyl acceptor [14]. In the case of human milk α-(1→3/4)-fucosyltransferase, the enzyme recognizes the oxygen atom at the 5′a position of 5′a-carba-D-Gal-β-(1→3)-GlcNAc, whereas the oxygen atom at the 5′a position of 5′a-carba-D-Gal-β-(1→4)-GlcNAc is not used for specific recognition by this fucosyltransferase [15]. Among these glycosyltransferases only fucosyltransferase recognizes the ring oxygen

for specific recognition. Therefore, further investigation is needed to determine, by use of 5a-carba-sugar analogues, whether other glycosyltransferases recognize the ring oxygen.

3. Experimental

General.—^{1}H, 1D-TOCSY, and ^{13}C NMR spectra were recorded with Bruker Avance 400 (303 K) and DMX 500 (298 K) spectrometers. The chemical shifts are presented in ppm and referenced to HOD (4.81 ppm) in D_2O as the internal standard. The chemical shifts in the ^{13}C NMR spectra are expressed in ppm and referenced to dioxane (67.80 ppm) in D_2O as the external standard. High-resolution mass spectra were recorded on a Shimazu/Kratos concept-IIH instrument under FAB conditions. Bovine β-(1→4)-galactosyltransferase (EC 2.4.1.90) and calf intestinal alkaline phosphatase (EC 3.1.3.1) were purchased from Sigma Chemical Co. and Boehringer Mannheim, respectively. UDP-α-[U-^{14}C]-D-galactose was purchased from NEN Research Products.

Enzyme assay of 5a-carba-sugar analogues.—Transfer velocity was measured under the following conditions. An assay solution containing UDP-[U-^{14}C]-galactose (9.92 GBq/mmol, 90 pmol), 5a-carba-DL-sugar analogues (20 mM), bovine serum albumin (0.1 μg), $MnCl_2$ (20 mM) and β-(1→4)-galactosyltransferase in HEPES buffer (100 mM, pH 7.0, total volume 30 μL) was incubated 37 °C for 60 min. The ^{14}C-labelled corresponding disaccharide analogues were isolated by passage of the mixture diluted with 1 mL of water through a Pasteur pipette column packed with Dowex 1-X8 (Cl^- form), and further eluted by 1 mL of water. The ^{14}C-labelled disaccharide analogue was measured by scintillation counting.

Inhibition assay with 5a-carba-DL-sugar analogues toward 2-acetamido-2-deoxy-β-D-glucopyranose was performed with a solution containing UDP-[U-^{14}C]-D-galactose (9.92 GBq/mmol, 90 pmol), 2-acetamido-2-deoxy-β-D-glucopyranose (3 mM), 5a-carba-DL-sugar analogue (20 mM), bovine serum albumin (0.1 μg), $MnCl_2$ (20 mM) and β-(1→4)-galactosyltransferase in HEPES buffer (100 mM, pH 7.0, total volume 30 μL). The [^{14}C]-labelled *N*-acetyl-D-lactosamine was quantitated in the same manner as above, and then the inhibition abilities of the 5a-carba-DL-sugars were determined.

Apparent kinetic parameters of β-(1→4)-galactosyltransferase toward synthetic 5a-carba-D-GlcNAc analogues were examined under the following conditions. An assay solution containing UDP-[U-^{14}C]-D-galactose (9.92 GBq/mmol, 90 pmol), 5a-carba-DL-GlcNAc (2.5, 5, 10, and 20 mM), bovine serum albumin (0.1 μg), $MnCl_2$ (20 mM) and β-(1→4)-galactosyltransferase in HEPES buffer (100 mM, pH 7.0, total volume 30 μL) was incubated at 37 °C. The reaction was followed up to 15% consumption of UDP-[U-^{14}C]-D-galactose. The corresponding ^{14}C-labelled *N*-acetyl-D-lactosamine analogues were isolated and measured in the same manner as above. The assay was performed in duplicate, and K_m and V_{max} were determined using a Lineweaver–Burk plot. The concentration of 5a-carba-D-GlcNAc was estimated as a half of the concentration of 5a-carba-DL-GlcNAc added to the assay mixture.

Enzymatic synthesis of N-*acetyl*-D-*lactosamine analogues.*—A galactosyl transfer reaction was performed using UDP-D-galactose disodium salt (1.5 equiv), 5a-carba-α-DL-GlcNAc (5.8 mg) or 5a-carba-β-DL-GlcNAc (6.0 mg) and β-(1→4)-galactosyltransferase (0.8 U) in HEPES buffer (100 mM, pH 7.0, total volume 1.0 mL) incubated at 37 °C for 48 h. The reactions afforded corresponding D-Gal-β-(1→4)-5a-carba-α-D-GlcNAc (**6**, 2.0 mg) and D-Gal-β-(1→4)-5a-carba-β-D-GlcNAc (**7**, 1.1 mg), which were purified on a silica gel column (3:2:1 EtOAc–MeOH–water), a gel-permeation column (Sephadex G-15, water), and by HPLC (Asashipak: GS-320, water) after deionization through an anion-exchange column (Dowex 1 × 2, Cl^- form).

Data for **6**: ^{1}H NMR (400.13 MHz): δ 4.58 (d, 1 H, *J* 7.8 Hz, H-1′), 4.16 (ddd, 1 H, *J* 2.5, 3.3, 2.9 Hz, H-1), 4.02 (d, 1 H, *J* 3.4 Hz, H-4′), 3.90–3.79 (m, 5 H, H-5′, 6a, 6b, 6′a, 6′b), 3.86 (dd, 1 H, *J* 8.1, 11.0 Hz, H-3), 3.81 (dd, 1 H, *J* 2.9, 11.0 Hz, H-2), 3.76 (dd, 1 H, *J* 3.4, 10.1 Hz, H-3′), 3.70 (dd, 1 H, *J* 8.1, 10.5 Hz, H-4), 3.65 (dd, 1 H, *J* 7.8, 10.0 Hz, H-2′), 2.20–2.10 (m, 1 H, H-5), 2.13 (s, 3 H, Ac),

1.98 (ddd, 1 H, *J* 3.3, 3.7, 14.6 Hz, H-7eq), 1.66 (ddd, 1 H, *J* 2.5, 14.6, 16.4 Hz, H-7ax); ^{13}C NMR (125.77 MHz): δ 175.28, 104.43 (C-1′), 85.10 (C-4), 76.55 (C-5′), 73.76 (C-3′), 72.36 (C-2′), 71.90 (C-3), 69.70 (C-4′), 68.12 (C-1), 62.73 (C-6), 62.09 (C-6′), 56.79 (C-2), 38.69 (C-5), 32.21 (C-7), 23.09 (Me); HRFABMS: Calcd for $C_{15}H_{27}NNaO_{10}$ [M + Na$^+$], 404.1533; Found: 404.1547.

Data for **7**: ^{1}H NMR (400.13 MHz): δ 4.54 (d, 1 H, *J* 7.7 Hz, H-1′), 3.99 (d, 1 H, *J* 3.5 Hz, H-4′), 3.89 (dd, 1 H, *J* 3.7, 11.3 Hz, H-6a), 3.85–3.77 (m, 4 H, H-5′, 6b, 6′a, 6′b), 3.74 (dd, 1 H, *J* 10.2, 10.2 Hz, H-2), 3.73 (dd, 1 H, *J* 3.5, 9.7 Hz, H-3′), 3.70 (dd, 1 H, *J* 8.9, 10.7 Hz, H-4), 3.66 (ddd, 1 H, *J* 4.5, 10.2, 11.3 Hz, H-1), 3.61 (dd, 1 H, *J* 7.7, 9.7 Hz, H-2′), 3.52 (dd, 1 H, *J* 8.9, 10.2 Hz, H-3), 2.12 (ddd, 1 H, *J* 4.5, 4.5, 12.6 Hz, H-7eq), 2.10 (s, 3 H, Ac), 1.91–1.81 (m, 1 H, H-5), 1.46 (ddd, 1 H, *J* 11.3, 12.6, 12.9 Hz, H-7ax); ^{13}C NMR (125.77 MHz): δ 175.89, 104.34 (C-1′), 84.20 (C-4), 76.57 (C-5′), 74.59 (C-3), 73.74 (C-3′), 72.33 (C-2′), 70.58 (C-1), 69.72 (C-4′), 62.55 (C-6), 62.12 (C-6′), 59.28 (C-2), 40.78 (C-5), 33.93 (C-7), 23.40 (Me); HRFABMS: Calcd for $C_{15}H_{27}NNaO_{10}$ [M + Na$^+$], 404.1533; Found: 404.1564.

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