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Note

Synthesis of α-Gal epitope derivatives with a galactosyltransferase–epimerase fusion enzyme

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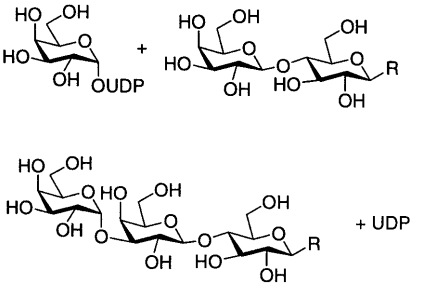
Abstract

α-Gal epitopes are carbohydrate structures bearing an α-D-Gal*p*-(1→3)-β-D-Gal*p* terminus and are the main cause of antibody-mediated hyperacute rejection in xenotransplantation. Nine monosaccharides and ten disaccharides were evaluated as substrates for a fusion protein, which contains both α-(1→3)-galactosyltransferase and uridine-5′-diphosphogalactose 4-epimerase. Four disaccharide and six trisaccharide α-Gal epitope derivatives were synthesized utilizing this novel fusion enzyme. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Epitopes; Fusion protein; Enzymatic glycosylation

1. Introduction

Hyperacute rejection (HAR) presents a formidable barrier to transplantation of organs from nonprimates to human (xenotransplantation) [1]. The major xenoactive antigens responsible for HAR have been identified as carbohydrate structures with a terminal α-D-Gal*p*-(1→3)-β-D-Gal*p* sequence (often termed α-Gal epitopes) [2]. The most common α-Gal epitopes are trisaccharides α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc, and pentasaccharide α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p* [3]. These epitopes are abundantly expressed on the cells of most mammals with the exception of catarrhines (humans, apes, and Old World monkeys) [4]. α-(1→3)-Galactosyltransferase [α-(1→3)-GalT, EC 2.4.1.151] is the enzyme responsible for the formation of the α-D-Gal(1→3)-β-D-Gal glycosidic bond in α-Gal epitopes in animals (Scheme 1) [5]. This enzyme is distinct from the human blood group B galactosyltransferase, which requires a fucosyl residue α-(1→2) linked to a β-linked galactose.



Scheme 1.

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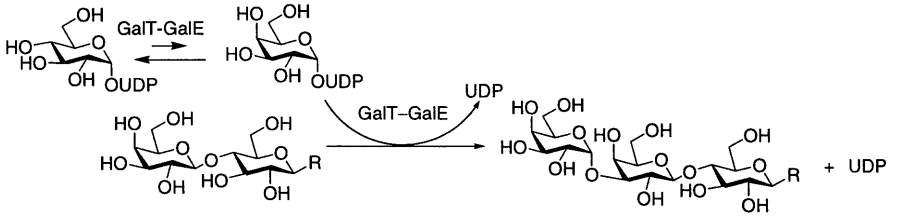


Scheme 2.

In the course of our studies on α-Gal epitopes we have cloned a truncated bovine α-(1→3)-GalT for the synthesis of α-Gal epitopes and their derivatives [6]. In order to reduce the cost associated with using expensive uridine 5′-diphosphogalactose (UDP-Gal), we have further constructed a fusion enzyme [7]. The *Escherichia coli galE* of gene uridine-5′-diphosphogalactose 4-epimerase (GalE, EC 5.1.3.2) was in-frame fused to the gene of 3′-terminus of the bovine α-(1→3)-galactosyltransferase (GalT) within a high-expression plasmid. The resultant enzyme exhibited both activities of GalE and GalT. The enzyme epimerized the inexpensive uridine 5′-diphosphoglucose (UDP-Glc) to UDP-Gal, then carried out the subsequent glycosylation (Scheme 2). For the synthesis of α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p* from UDP-glucose and lactose, GalT–GalE exhibited kinetic advantage with an overall reaction rate 300% higher than that of the system containing equal amounts of epimerase and galactosyltransferase [7]. This paper reports the application of this enzyme in the synthesis of α-Gal epitopes and their derivatives. Various monosaccharides and disaccharides were evaluated as substrates for GalT–GalE catalyzed α-galactosylation. It was found that GalT–GalE could accept a broad spectrum of substrates. Ten α-Gal derivatives and analogs were synthesized on a preparative scale. All these products have the potential to be inhibitors of anti-α-Gal antibodies.

2. Results and discussion

Relative activity assay.—Nine monosaccharides (**1**–**9**) and ten disaccharides (**10**–**19**) were evaluated as substrates in a radiochemical assay. The relative activities of the enzyme towards the monosaccharides are shown in Table 1. It was found that methyl galactose exhibited 9% activity and therefore could be considered as an acceptable substrate for GalT–GalE. This indicates that the glucose or

Table 1
Relative activities of monosaccharides with the GalT–GalE enzyme

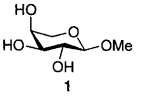
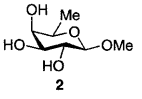
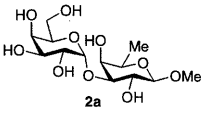
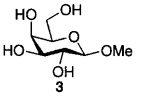
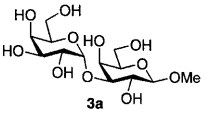
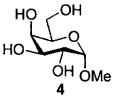
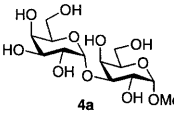
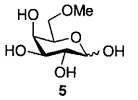
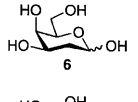
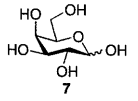
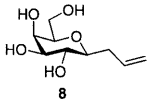
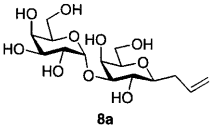
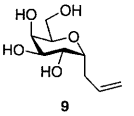
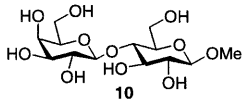
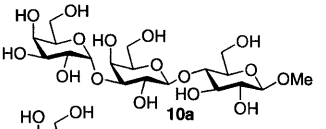
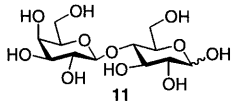
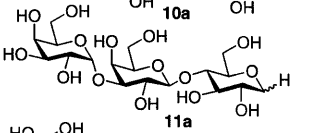
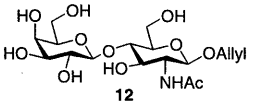
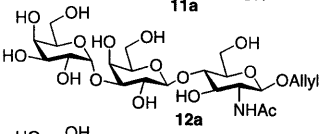
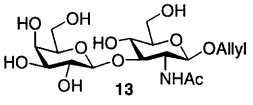
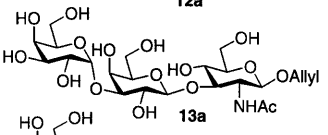
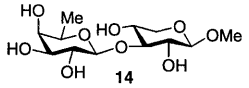
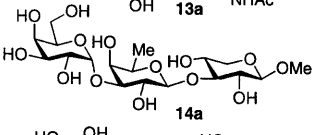
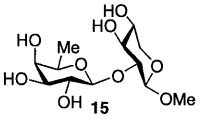
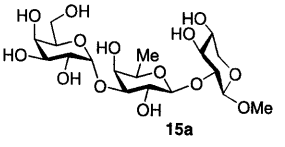
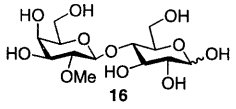
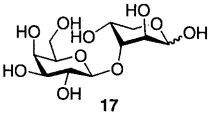
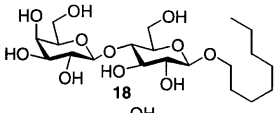
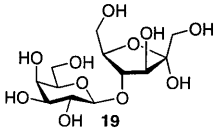
Substrate	Relative activity	Yield	Product
 **1**	0	NA	NA
 **2**	3%	6%	 **2a**
 **3**	9%	18%	 **3a**
 **4**	2%	5%	 **4a**
 **5**	0	NA	NA
 **6**	0	NA	NA
 **7**	3%	NA	NA
 **8**	5%	11%	 **8a**
 **9**	1%	NA	NA

Table 2
Relative activities of disaccharides with the GalT–GalE enzyme

Substrate	Relative activity	Yield	Product
 10	100%	56%	 10a
 11	61%	51%	 11a
 12	155%	66%	 12a
 13	43%	49%	 13a
 14	16%	19%	 14a
 15	17%	18%	 15a
 16	6%	NA	NA
 17	19%	NA	NA
 18	57%	NA	NA
 19	29%	NA	NA

2-acetamido-2-deoxy-D-glucose ('*N*-acetylglucosamine') residue of the natural substrates aids the binding but is not crucial. The enzyme activity is influenced by the anomeric configuration of the substrate. The β isomer seems to be the favored form in this type of glycosylation. This finding can explain why methyl β-D-galactopyranoside (**3**) has a higher activity than free galactose (**7**), which favors the α isomer in aqueous solution. Methyl β-D-fucopyranoside (**2**) had one-third of the activity of compound **3**, clearly indicating that a 6′-OH was not an absolute requirement for binding, while methyl β-D-arabinopyranoside (**1**) did not exhibit any activity. One explanation for this finding is that the 6-methylene group of D-galactose or the 6-methyl group of D-fucose might be required to bind to some

hydrophobic site of the enzyme. Two C-linked galactose derivatives **8** and **9** were also evaluated as substrates. β-Linked **8** had higher activity than its α-linked isomer **9**.

Ten disaccharides, two of which had D-fucose and the other eight had D-galactose as the non-reducing terminus (Table 2), were evaluated. For the two natural substrates **10** and **12**, *N*-acetyllactosamine derivative **12** was shown to be the better substrate. The result agrees with the findings by Sujino et al. who used calf thymus α-(1→3)-galactosyltransferase [8]. When the linkage of galactose and *N*-acetylglucosamine was changed from β-(1→4) (compound **12**) to β-(1→3) (compound **13**), GalT–GalE exhibited one fourth of the activity. This is in stark contrast to the result with calf thymus α-(1→3)-galactosyltransferase, in which only 4% activity was observed [8]. One noteworthy observation is that the enzyme exhibited 16% and 17% activities toward compounds **14** and **15**, both of which are constructed from D-fucose and D-xylose either with a β-(1→3) or β-(1→2) linkage.

Preparative synthesis.—Ten compounds, four disaccharides and six trisaccharides, were synthesized in preparative scale utilizing GalT–GalE in the presence of UDP-Glc as glycosylation donor (Tables 1 and 2). The yields corresponded to the activity of the enzyme, ranging from 5 to 66%.

3. Experimental

General.—^{1}H and ^{13}C NMR spectra were recorded on a 400 or 500 MHz spectrometer. Thin-layer chromatography (TLC) was conducted on J.T. Baker Si_{250F} silica gel TLC plates with a fluorescent indicator. Gel-filtration chromatography was performed using Sephadex G-15. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. Compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **10**, **11**, **17**, and **19** were purchased from commercial sources. Two *C*-glycosyl type galactose derivatives **8** and **9** were synthesized from galactose [9]. Compound **12** was prepared in four steps from lactosamine [10]. Compound **13** was synthesized chemically by coupling D-galactose and *N*-acetylglucosamine [11]. Compounds **15** and **16** were products of thermophilic glycosidase-catalyzed glycosylation [12]. Compound **18** was prepared from lactose that was coupled with octanol. All synthesized compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, as well as by mass spectrometry.

Relative activity assay.—A standard assay solution contained the following components: 10 mM $MnCl_2$, 0.1% BSA, 0.3 mM UDP-D-[6-^{3}H] glucose (final specific activity of 1000 cpm/nmol), 20 μL of purified GalE–GalT in 10 mM Tris–HCl buffer (pH 7.0), and 20 mM of one of the various substrates. The final volume was adjusted to 100 μL with Tris–HCl buffer (pH 7.0). A solution containing all components except the substrate was used as a blank. The reaction was conducted at 37 °C for 15 min, and was terminated by adding 900 μL of ice-cold EDTA (0.01 M). Unreacted radiolabeled donor was removed with anion-exchange resin (Dowex 1 × 8-200, Cl^- form). The radioactivity was quantitated by a liquid scintillation counter (Beckman LS-3801) in ScintiVerse BD scintillation cocktail (5 mL).

General procedure for preparative synthesis.—GalE–GalT was added to a Tris–HCl buffer (100 mM, pH 7.0) solution of substrate (40 mM), UDP-glucose (44 mM), $MnCl_2$ (10 mM) and BSA (0.1%). The mixture was agitated under argon at rt for 3 days, and the product was separated by gel-filtration chromatography.

Methyl α-D-galactopyranosyl-(1→3)-β-D-fucopyranoside (**2a**) *(4.5 mg, 6%).*—^{1}H NMR (D_2O): δ 4.97 (d, *J* 4.0 Hz, 1 H), 4.18 (d, *J* 8.0 Hz, 1 H), 4.00 (t, *J* 5.5 Hz, 1 H), 3.82 (t, *J* 3.5 Hz, 1 H), 3.78 (dd, *J* 3.0, 10.5 Hz, 2 H), 3.67 (dd, *J* 4.0, 10.5 Hz, 1 H), 3.62–3.55 (m, 4 H), 3.42 (dd, *J* 1.5, 8.0 Hz, 1 H), 3.38 (s, 3 H), 1.11 (d, *J* 6.5 Hz, 3 H); ^{13}C NMR (D_2O): δ 103.7, 95.3, 77.5, 71.1, 70.9, 69.5, 69.4, 69.1, 68.5, 67.6, 61.2, 57.3, 15.6; FABMS: 363 $(M + Na)^+$.

Methyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranoside (**3a**) *(35 mg, 18%).*—^{1}H NMR (D_2O): δ 4.99 (d, *J* 4.2 Hz, 1 H), 4.22 (d, *J* 7.8 Hz, 1 H), 4.02–3.99 (m, 2 H), 3.85 (d, *J* 3.0 Hz, 1 H), 3.81–3.45 (m, 9 H), 3.42 (s, 3 H); ^{13}C NMR (D_2O): δ 103.7, 95.3, 77.4, 75.0, 71.0, 69.4, 69.3, 69.3, 68.3, 64.9, 61.1, 57.3; FABMS: 357 $(M + H)^+$.

Methyl α-D-galactopyranosyl-(1→3)-α-D-galactopyranoside (**4a**) *(5.0 mg, 5%).*—^{1}H NMR (D_2O): δ 5.11 (d, *J* 4.0 Hz, 1 H), 4.84 (d, *J* 4.0 Hz, 1 H), 4.18 (d, *J* 2.4 Hz, 1 H), 4.13 (t, *J* 6.0 Hz, 1 H), 3.97 (d, *J* 3.2 Hz, 1 H), 3.92 (d, *J* 3.6 Hz, 1 H), 3.90 (t, *J* 2.4 Hz, 1 H), 3.86–3.77 (m, 2 H), 3.73 (d, *J* 2.4 Hz, 1 H), 3.70 (t, *J* 5.6 Hz, 4 H); ^{13}C NMR (D_2O): δ 99.5, 95.1, 74.2, 71.0, 70.7, 69.4, 69.3, 68.3, 66.68, 65.5, 61.3, 61.1, 55.1; FABMS: 357 $(M + H)^+$.

α-D-Galactopyranosyl-(1→3)-β-D-galactopyranosyl-1-propene (**8a**) *(11 mg, 11%).*—^{1}H NMR (D_2O): δ 5.88 (m, 1 H), 5.13–5.04 (m, 2 H), 5.07 (d, *J* 3.2 Hz, 1 H), 4.11 (m, 2 H), 3.93 (d, *J* 2.4 Hz, 1 H), 3.88–3.75 (m, 3 H), 3.67–3.51 (m, 6 H), 3.33 (m, 1 H), 2.56 (m, 1 H), 2.24 (m, 1 H); ^{13}C NMR (D_2O): δ 134.9, 117.5, 95.2, 79.3, 78.7, 78.5, 70.9, 69.6, 69.3, 68.7, 68.5, 65.5, 61.4, 61.0, 35.6; FABMS: 389 $(M + Na)^+$.

Methyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (**10a**) *(22 mg, 56%).*—^{1}H NMR (D_2O): δ 4.96 (d, *J* 4.2 Hz, 1 H), 4.33 (d, *J* 7.5 Hz, 1 H), 4.23 (d, *J* 4.23 Hz, 1 H), 3.99 (m, 2 H), 3.82–3.44 (m, 15 H), 3.38 (s, 3 H), 3.11 (m, 1 H); ^{13}C NMR (D_2O): δ 103.2, 102.9, 95.5, 78.6, 77.2, 75.2, 74.8, 74.6, 72.9, 70.9, 69.7, 69.4, 69.2, 68.3, 64.9, 61.1, 61.0, 60.18, 57.3; FABMS: 519 $(M + H)^+$.

Allyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**12a**) *(23 mg, 66%).*—^{1}H NMR (D_2O): δ 1.84 (s, 3 H), 3.38–4.02 (m, 19 H), 4.15 (dd, *J* 5.0, 13.0 Hz, 1 H), 4.35 (d, *J* 8.0 Hz, 1 H), 4.39 (d, *J* 8.5 Hz, 1 H), 4.95 (d, *J* 3.5 Hz, 1 H), 5.06–5.14 (m, 2 H), 5.67–5.75 (m, 1 H); ^{13}C NMR (D_2O): δ 22.0, 54.9, 60.0, 60.8, 60.9, 64.7, 68.1, 69.0, 69.2, 69.5, 70.4, 70.7, 72.4, 74.6, 74.9, 77.0, 78.5, 95.3, 99.9, 102.7, 118.1, 133.1, 174.5; FABMS: 586 $(M + H)^+$.

Allyl α-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (**13a**) *(45mg, 49%).*—^{1}H NMR (D_2O): δ 5.87–5.79 (m, 1 H), 5.22 (m, 2 H), 5.06 (d, 3.2 Hz, 1 H), 4.42 (d, *J* 8.4 Hz, 1 H), 4.29 (d, *J* 8.4 Hz, 1 H), 4.15 (dd, *J* 4.5, 13.2, 1 H), 3.98 (m, 3 H), 3.81–3.29 (m, 11 H), 1.94 (s, 3 H). ^{13}C NMR (D_2O): δ 133.6, 118.5, 103.7, 100.1, 95.7, 83.4, 77.6, 75.6, 75.3, 71.1, 70.7, 69.6, 69.4, 69.1, 68.5, 65.0, 61.3, 61.3, 61.0, 54.6, 22.5; FABMS: 608 $(M + Na)^+$.

Methyl α-D-galactopyranosyl-(1→3)-β-D-fucopyranosyl-(1→3)-β-D-xylopyranoside (**14a**) *(30 mg, 19%).*—^{1}H NMR (D_2O): δ 4.99 (d, *J* 4.5 Hz, 1 H), 4.52 (d, *J* 7.5 Hz, 1 H), 4.21 (d, *J* 7.5 Hz, 1 H), 4.05 (t, *J* 6.5 Hz, 1 H), 3.88–3.80 (m, 4 H), 3.71–3.62 (m, 3 H), 3.58–3.49 (m, 5 H), 3.39 (s, 3 H), 3.30 (t, *J* 8.0 Hz, 1 H), 3.20 (dd, *J* 10.0, 11.5 Hz, 1 H), 1.13 (d, *J* 7.0 Hz, 3 H); ^{13}C NMR (D_2O): δ 103.8, 103.1, 96.4, 84.6, 77.3, 72.5, 71.0, 70.8, 69.6, 69.5, 69.3, 68.4, 68.1, 67.5, 64.7, 61.1, 57.3, 15.6; FABMS: 495 $(M + Na)^+$.

Methyl α-D-galactopyranosyl-(1→3)-β-D-fucopyranosyl-(1→2)-β-D-xylopyranoside (**15a**) *(37 mg, 18%, contaminated by ca. 10% of unknown compound).*—^{1}H NMR (D_2O): δ 4.98 (d, *J* 4.0 Hz, 1 H), 4.51 (d, *J* 7.5 Hz, 1 H), 4.30 (d, *J* 7.5 Hz, 1 H), 4.03 (t, *J* 6.0 Hz, 1 H), 3.85–3.79 (m, 3 H), 3.68 (dd, *J* 4.0, 10.5 Hz, 1 H), 3.63–3.56 (m, 4 H), 3.51–3.45 (m, 3 H), 3.36 (s, 3 H), 3.21–3.16 (m, 1 H), 1.12 (d, *J* 7.0 Hz, 3 H); ^{13}C NMR (D_2O): δ 103.2, 102.8, 95.37, 80.6, 77.4, 75.0, 71.0, 70.9, 69.8, 69.5, 69.3, 69.1, 68.4, 67.6, 64.7, 61.1, 57.0, 15.7; FABMS: *m/z* 473 $(M + H)^+$.

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