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Acceptor hydroxyl group mapping for calf thymus α-(1 → 3)-galactosyltransferase and enzymatic synthesis of α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β D-Glc*p*NAc analogs

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Abstract

The epitope of the acceptor substrate for α-(1 → 3)-galactosyltransferase from calf thymus has been mapped by using a series of mono-deoxygenated and mono-*O*-alkylated Type II (β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc) disaccharides. The 4-OH group of the β-D-galactopyranosyl residue is a key polar group essential for glycosyl transfer, tolerating neither deoxygenation nor *O*-alkylation. Substitution at positions 6 and 6′ by a variety of polar alkyl substituents was readily tolerated, allowing the preparative enzymatic synthesis of a series of trisaccharide derivatives carrying polar substituents on each of these hydroxyl groups. These new analogs are potential inhibitors of *Clostridium difficile* toxin A and of a human anti-α-Gal antibody. © 1998 Elsevier Science Ltd.

Keywords: α-(1 → 3)-Galactosyltransferase; α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc, analogs

1. Introduction

α-(1 → 3)-Galactosyltransferase (α-(1 → 3) GalT, E.C. 2.4.1.151) catalyzes the transfer of D-galactopyranose from UDP-Gal to the 3′ hydroxyl group of β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc–OR to yield α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-OR (Scheme 1). This enzyme has garnered interest because of the discovery of a naturally occuring human antibody to glycoconjugates bearing a non-reducing terminal α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc–OR sequence [1–4].

The interaction between oligosaccharides and proteins, including enzymes, is frequently based on the recognition of only a few of the hydroxyl groups on the carbohydrate moiety [5–7]. In this study, the identity of these key hydroxyl groups on the acceptor for α-(1 → 3) GalT isolated from calf thymus was established utilizing monodeoxygenated and monosubstituted β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc–OR derivatives in which R are alkyl groups. The ability of α-(1 → 3) GalT to glycosylate analogs permitted

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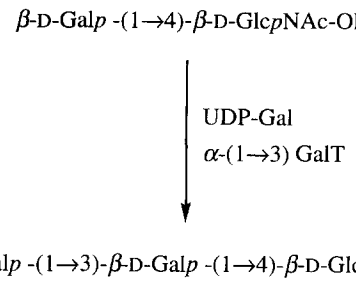


Scheme 1. Synthesis of α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-OR catalyzed by α-(1 → 3)-Galactosyltransferase.

the preparative enzymatic synthesis of 10 analogs of this trisaccharide carrying polar substituents on the hydroxyl groups. These new α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc–OR analogs are potential inhibitors of both the anti-α-Gal antibody and toxin A of *Clostridium difficile* which is the causative agent of pseudomembranous colitis [2].

2. Results and discussion

Enzymatic assays.—A series of monodeoxygenated Type II (β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc) derivatives (**3**, **8**, **13**, **18**, **23**), as well as Type II derivatives bearing four kinds of polar functional groups (**4–7**, **9–12**, **14–17**, **19–22**, **24–27**) [8] were initially screened as potential acceptors for the α-(1 → 3) GalT. The lactose derivative **2** and both natural and monodeoxygenated Type I (β-D-Gal*p*-(1 → 3)-β-D-Glc*p*NAc) derivatives (**28**, **29**) were also evaluated. The relative rates of galactosylation are shown in Table 1 where they are compared against the unmodified Type II acceptor **1**. The conclusions of this screening are that: (1) both deoxygenation and *O*-substitution were tolerated at OH-6, 2′, and 6′ of Type II derivatives; (2) only deoxygenation was tolerated at OH-3 of Type II derivatives; (3) neither deoxygenation nor substitution were tolerated at the OH-4′ of Type II derivatives. These results demonstrate that the OH-4′ of Type II acceptors is a key polar group for the α-(1 → 3) GalT; (4) although Type I structures are known to be substrates [9], the 4-deoxy Type I derivative **29** was found to be equally active. These results are summarized schematically in Fig. 1.

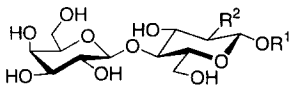
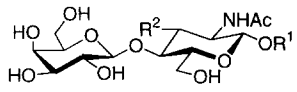
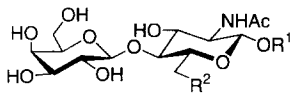
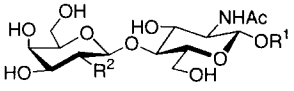
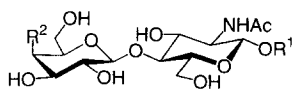
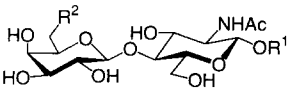
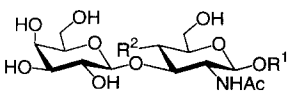
Preparative synthesis of trisaccharide analogs.—The eleven natural and modified disaccharides which were found to be substrates for α-(1 → 3) GalT were preparatively galactosylated utilizing this enzyme in the presence of the donor of UDP-Gal (Scheme 1 and Table 2). In these reactions, UDP is produced as the reaction progresses. Since it is a potent inhibitor of this enzyme, alkaline phosphatase was added to degrade UDP to uridine, which is less-inhibitory. In every case, TLC showed the formation of a single product which was easily isolated by sequential column chromatography on reverse-phase C_{18} silica and Iatrobeads. The yields ranged from 35 to 97%. The details of the syntheses are summarized in Table 3. All products were stable to purification except for **35** which was isolated along with the amide derivative **34** as a result of hydrolysis of the amidine group. The structures of the products were confirmed by their ^{1}H NMR and mass spectral data (Table 4). The characteristic H-1 protons of galactose in α-configuration were present at 5.1–5.2 ppm with coupling constants of 3.6–4.0 Hz as expected [10,11]. The mass spectra confirmed that a single galactosyl residue had been added to each acceptor. Evaluation of the toxin binding ability of these products is in progress and the results will be reported elsewhere.

3. Experimental

Materials.—Calf thymus α-(1 → 3) GalT was isolated according to literature procedures [12]. Compounds **1**, **2**, **3**, **13**, **28** and **29** were synthesized as previously described [13,14]. Compounds **4–7**, **9–12**, **14–17**, **19–22**, **24–27** were also synthesized as previously described [8]. Compounds **8**, **18** and **23** were generous gifts from R.U. Lemieux. UDP-Gal (Na^+ salt) was from Sigma. Alkaline phosphatase (1 U/μL) from calf intestine was from Boehringer Mannheim. EcoLite (+) was from ICN. TLC was conducted on glass plates precoated with 250 μm layers of Silica Gel $60F_{254}$ (E. Merck, Darmstadt). 'Iatrobeads' refers to a beaded silica gel (Product No. 6RS-8060) from Iatron Laboratories (Tokyo). C_{18} Sep-Pak cartridges were from Waters. Millex-GV filters (0.22 μm) were from Millipore. ^{1}H NMR spectroscopy was performed on a Bruker AMR-360 (360 MHz) operating at ambient temperature. For **30**, a Varian UNITY 500 (500 MHz) instrument was used. Only partial NMR data are reported and the remaining data were in accordance with the proposed structures. HRMS spectra were recorded on a Micromass ZabSpec Hybrid Sector-TOF using a 1% solution of CH_3COOH in 1:1 water:MeOH as the liquid carrier.

Radiochemical assay.—Standard enzyme assays contained the following components: a disaccharide

Table 1
Relative rates of α-(1 → 3) GalT catalyzed glycosylation

		R^1	R^2	relative rate[a] (%)
	**1**	$(CH_2)_8CO_2Me$	NHAc	100
	2	$(CH_2)_8CO_2Me$	OH	31
	**3**	$(CH_2)_8CO_2Me$	H	55
	4	$(CH_2)_7CH_3$	$OCH_2CH_2NH_2$	<1
	5	$(CH_2)_7CH_3$	OCH_2CO_2H	<1
	6	$(CH_2)_7CH_3$	OCH_2CONH_2	<1
	7	$(CH_2)_7CH_3$	$OCH_2C(NH_2)_2^+Cl^-$	<1
	**8**	Me	H	76
	9	$(CH_2)_7CH_3$	$OCH_2CH_2NH_2$	11
	10	$(CH_2)_7CH_3$	OCH_2CO_2H	54
	11	$(CH_2)_7CH_3$	OCH_2CONH_2	56
	12	$(CH_2)_7CH_3$	$OCH_2C(NH_2)_2^+Cl^-$	10
	**13**	$(CH_2)_8CO_2Me$	H	25
	14	$(CH_2)_7CH_3$	$OCH_2CH_2NH_2 \cdot HCl$	5
	15	$(CH_2)_7CH_3$	OCH_2CO_2H	1
	16	$(CH_2)_7CH_3$	OCH_2CONH_2	1
	17	$(CH_2)_7CH_3$	$OCH_2C(NH_2)_2^+Cl^-$	1
	**18**	$(CH_2CH_2O)_2CH_2CO_2Me$	H	<1
	19	$(CH_2)_7CH_3$	$OCH_2CH_2NH_2 \cdot HCl$	1
	20	$(CH_2)_7CH_3$	OCH_2CO_2H	<1
	21	$(CH_2)_7CH_3$	OCH_2CONH_2	<1
	22	$(CH_2)_7CH_3$	$OCH_2C(NH_2)_2^+Cl^-$	<1
	**23**	$(CH_2CH_2O)_2CH_2CO_2Me$	H	39
	24	$(CH_2)_7CH_3$	$OCH_2CH_2NH_2 \cdot HCl$	20
	25	$(CH_2)_7CH_3$	OCH_2CO_2H	7
	26	$(CH_2)_7CH_3$	OCH_2CONH_2	7
	27	$(CH_2)_7CH_3$	$OCH_2C(NH_2)_2^+Cl^-$	5
	**28**	$(CH_2)_8CO_2Me$	OH	4
	29	$(CH_2)_8CO_2Me$	H	3

[a]The concentration of each analog was 540 μM.

analog (10.8 nmol), UDP-Gal (10 nmol), UDP-[6-^{3}H]Gal (about 100,000 dpm), 4 μL of assay buffer (500 mM sodium cacodylate, 250 mM manganese(II) chloride, 4% Triton X-100, 5 mg/mL BSA, pH 6.0), 7 μL of buffer (30 mM sodium cacodylate, 20 mM manganese(II) chloride, 0.1% Triton X-100, pH 6.5), and enzyme solution (30 mM sodium cacodylate, 20 mM manganese(II) chloride, 0.3% Triton X-100, pH 6.5, 8 μL, 50 μU) and water to 20 μL final volume. Reaction mixtures were incubated for 30 min at 37 °C, diluted with water to 200 μL and loaded onto a C_{18} Sep-Pak cartridge which was pre-equilibrated with MeOH (10 mL) and water (10 mL). The cartridge was washed with water (50 mL) and the product was eluted with MeOH (4 mL). The radioactivity of the MeOH eluates were quantitated by liquid scintillation in EcoLite (+) scintillation cocktail (10 mL). For compound **8**, which did not have a hydrophobic aglycone, anion exchange resin (Bio-Rad AG 1 × 8 resin, 50–100 mesh, chloride form) was used to remove the unreacted radiolabeled donor from reaction product.

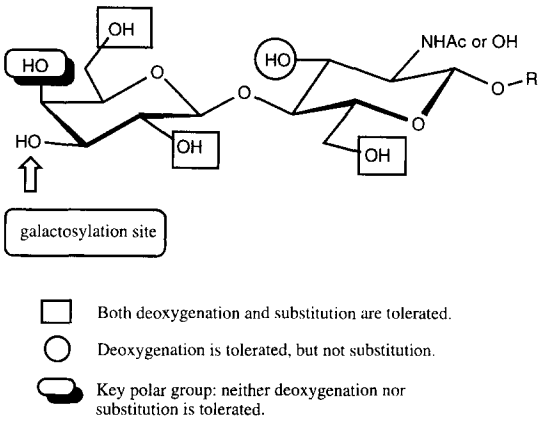
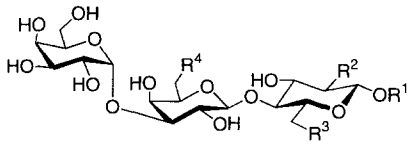


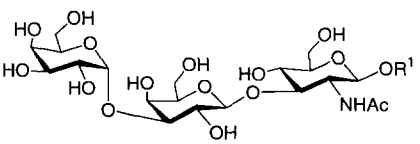
Fig. 1. Structural requirements of Type II acceptors for calf thymus α-(1 → 3) GalT.

Representative preparative synthesis.—Disaccharide **11** (2.2 mg, 4.0 nmol), UDP-Gal (1.2 mg, 1.9 nmol), and alkaline phosphatase (1 U/μL, 10 μL) were combined with a solution of α-(1 → 3) GalT (9 mU/mL, 1 mL) containing 30 mM sodium cacody-

Table 2
Structure of galactosylated products



	R^1	R^2	R^3	R^4
30	$(CH_2)_8CO_2Me$	NHAc	OH	OH
31	$(CH_2)_8CO_2Me$	OH	OH	OH
32	$(CH_2)_7CH_3$	NHAc	$OCH_2CH_2NH_2$	OH
33	$(CH_2)_7CH_3$	NHAc	OCH_2CO_2H	OH
34	$(CH_2)_7CH_3$	NHAc	OCH_2CONH_2	OH
35	$(CH_2)_7CH_3$	NHAc	$OCH_2C(NH_2)_2^+Cl^-$	OH
36	$(CH_2)_7CH_3$	NHAc	OH	$OCH_2CH_2NH_2$·HCl
37	$(CH_2)_7CH_3$	NHAc	OH	OCH_2CO_2H
38	$(CH_2)_7CH_3$	NHAc	OH	OCH_2CONH_2
39	$(CH_2)_7CH_3$	NHAc	OH	$OCH_2C(NH_2)_2^+Cl^-$



40	$(CH_2)_8CO_2Me$	

Table 3
Summary of reaction conditions for preparative enzymatic glycosylation

Starting material (mg)	UDP-Gal (eq./SM)	Enzyme[a] (mU)	Volume[b] (mL)	Reaction temperature and time	Product (mg)	Yield (%)
1 (10.0)	2.4	45	5.0	37 °C, 67 h	**30** (11.6)	90
2 (3.0)	4.4	51	1.4	37 °C, 191 h	**31** (3.0)	76
9 (2.2)	3.9	11	1.3	37 °C, 121 h	**32** (1.8)	63
10 (2.3)	2.8	9	1.0	37 °C, 72 h	**33** (2.9)	97
11 (2.2)	3.0	9	1.0	37 °C, 92 h	**34** (2.7)	94
12 (2.4)	3.2	11	1.2	37 °C, 146 h	**35** (1.2)	38
24 (2.8)	4.1	208	2.7	22 °C, 439 h	**36** (1.3)	35
25 (3.0)	4.8	300	3.9	37 °C, 61 h, r.t. 366 h	**37** (1.2)	41
26 (2.8)	2.1	56	2.1	37 °C, 50 h, r.t. 67 h	**38** (3.5)	96
27 (3.2)	1.3	92	1.2	22 °C, 233 h	**39** (2.7)	67
28 (2.0)	9.7	44	1.2	37 °C, 45 h, r.t. 233 h	**40** (1.9)	73

[a]Values represent a single aliquot of enzyme added to samples **10** and **11**, and the sum of several aliquots added to each of the other samples.
[b]Final volume of reaction mixture.

Table 4
Selected ^{1}H NMR and HRMS data for trisaccharides **30–40**

	Selected ^{1}H NMR data[a]						HRMS			R_f^e
	Ref.[b]	GlcNAc H-1[c]	NHAc	Galβ4 H-1[c]	Galα3 H-1	$R^1CH_2R^{2d}$	Calcd. for	*m/z*	Found	
30	A	4.52–4.54	2.04	4.55 (7.8)	5.15 (4.0)	–	$C_{30}H_{54}NO_{18}$	716.3341	716.3345	0.20[1]
31	B	4.52 (7.8)[f]	–	4.48 (8.0)	5.14 (3.8)	–	$C_{28}H_{50}O_{18}$	675.3075	675.3070	0.09[2]
32	A	4.52 (7.7)	2.06	4.55 (7.8)	5.17 (3.8)	2.99–3.04	$C_{30}H_{57}N_2O_{16}$	701.3708	701.3710	0.67[3]
33	A	4.56 (7.3)	2.06	4.60 (7.8)	5.17 (3.8)	3.76	$C_{30}H_{54}NO_{18}$	716.3341	716.3345	0.21[4]
34	B	4.50 (7.8)	2.06	4.54 (7.8)	5.14 (3.8)	4.12	$C_{30}H_{55}N_2O_{17}$	715.3501	715.3506	0.21[5]
35	A	4.51 (7.5)	2.06	4.57 (7.9)	5.17 (3.9)	4.52	$C_{30}H_{56}N_3O_{16}$	714.3669	714.3659	0.22[4]
36	A	4.56 (7.8)	2.06	4.57 (7.9)	5.16 (3.8)	3.14–3.17	$C_{30}H_{57}N_2O_{16}$	701.3708	701.3703	0.27[4]
37	A	4.52–4.56	2.06	4.59 (7.8)	5.18 (3.8)	3.77	$C_{30}H_{54}N_1O_{18}$	716.3341	716.3343	0.36[6]
38	A	4.54–4.57	2.06	4.58 (8.4)	5.16 (3.6)	4.12 (0.92)	$C_{30}H_{55}N_2O_{17}$	715.3501	715.3499	0.24[4]
39	A	4.55 (8.2)	2.06	4.58 (8.8)	5.16 (3.9)	4.51	$C_{30}H_{56}N_3O_{16}$	714.3661	714.3665	0.19[4]
40	B	4.55 (7.8)	2.02	4.49 (7.7)[g]	5.14 (3.9)	–	$C_{30}H_{54}NO_{18}$	716.3341	716.3340	0.08[2]

[a]Numbers in parentheses give coupling constants in Hz.
[b]A: Me in octyl group was used as a reference, 0.885; B: Acetone = 2.225.
[c]May be interchangeable.
[d]For **32** $OCH_2CH_2NH_2$; for **33** and **37** OCH_2COOH; for **34** and **38** OCH_2CONH_2; for **35** and **39** $OCH_2C(NH_2)_2^+Cl^-$; for **36** $OCH_2CH_2NH_2 \cdot HCl$.
[e]1 65:35:1 CH_2Cl_2–MeOH–H_2O.
[2]40:10:1 EtOAc–MeOH–H_2O.
[3]3:5:3 5% NH_3 in H_2O–MeOH–CH_2Cl_2.
[4]65:35:5:2 CH_2Cl_2–MeOH–H_2O–AcOH.
[5]130:70:1 CH_2Cl_2–MeOH–H_2O.
[6]30:25:7:3 EtOAc–MeOH–H_2O–AcOH.
[f]Glc H-1.
[g]Galβ3 H-1.

late, 20 mM manganese(II) chloride, 0.1% Triton X-100, pH 6.5. This mixture was incubated at 37 °C for 4 days. Five additional aliquots of UDP-Gal (total 10 nmol) were added during the incubation period. When TLC (130:70:1 CH_2Cl_2–MeOH–water) showed the complete disappearance of the starting acceptor, the solution was filtered through glass wool and loaded onto a C_{18} Sep-Pak cartridge which was pre-equilibrated with MeOH (10 mL) then water (10 mL). The cartridge was washed with water (40 mL) and product was eluted with MeOH (60 mL). The MeOH eluate was concentrated and the resulting residue was loaded onto a column of Iatrobeads (0.98 g) which was eluted with 9:1 CH_2Cl_2–MeOH, then 130:70:1 CH_2Cl_2–MeOH–water (15 mL). The fractions containing product were collected and concentrated. The residue was loaded onto a C_{18} Sep-Pak cartridge and the cartridge was washed with water (15 mL) then MeOH (25 mL). The MeOH eluate was concentrated and the product was dissolved in water (10 mL). This solution was passed through a Millex-GV filter (0.22 μm), and the filtrate was lyophilized to yield a fluffy white powder (**34**, 2.7 mg, 3.8 μmol, 94%).

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