ELSEVIER

Carbohydrate Research 276 (1995) 91–98

CARBOHYDRATE RESEARCH

Methyl 3-amino-3-deoxy-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside: an inhibitor of UDP-D-galactose: β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucose (1 → 3)-α-D-galactopyranosyltransferase ☆

Anne-Charlotte Helland [a], Ole Hindsgaul [a,*], Monica M. Palcic [a], Cheryl L.M. Stults [b], Bruce A. Macher [b]

[a] *Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada*

[b] *Department of Chemistry and Biochemistry, San Francisco State University, 1600 Holloway Avenue, San Francisco, CA 94132, USA*

Received 23 May 1994; accepted 10 March 1995

Abstract

UDP-D-galactose: β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucose α-(1 → 3)-D-galactopyranosyltransferase [E.C. 2.4.1.151] transfers D-galactosyl-residues from the sugar nucleotide with retention of configuration. We report here that synthetic methyl 3′-amino-3′-deoxy-*N*-acetyllactosaminide (**9**), where the hydroxyl group normally undergoing galactosylation has been replaced by amino group, is an inhibitor for this enzyme with $K_i = 104$ μM. The mode of inhibition is not competitive, but appears to be specific, since other glycosyltransferases were not affected by **9**.

☆ Trivial names include the following: methyl 3′-amino-3′-deoxy-*N*-acetyllactosaminide and α-(1 → 3)-galactosyltransferase or UDP-galactose: *N*-acetyllactosaminide α-(1 → 3)-galactosyltransferase or α-(1 → 3)-GalT.

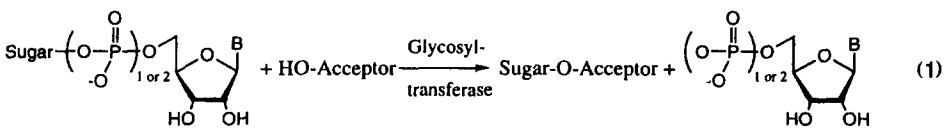
* Corresponding author.

Elsevier Science Ltd.
SSDI 0008-6215(95)00165-4

Keywords: UDP-D-Galactose:β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucose (1 → 3)-α-D-galactopyranosyltransferase; "α-(1 → 3)-Galactosyltransferase"; E.C. 2.4.1.151; Enzyme inhibitor; Methyl 3-amino-3-deoxy-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside

1. Introduction

Glycosyltransferases catalyze the transfer of single sugar residues from their sugar nucleotides to acceptor oligosaccharides (below, B = uracil, guanine or cytosine) thereby controlling the biosynthesis of mammalian glycoconjugates [1–4]. Specific inhibitors of glycosyltransferases



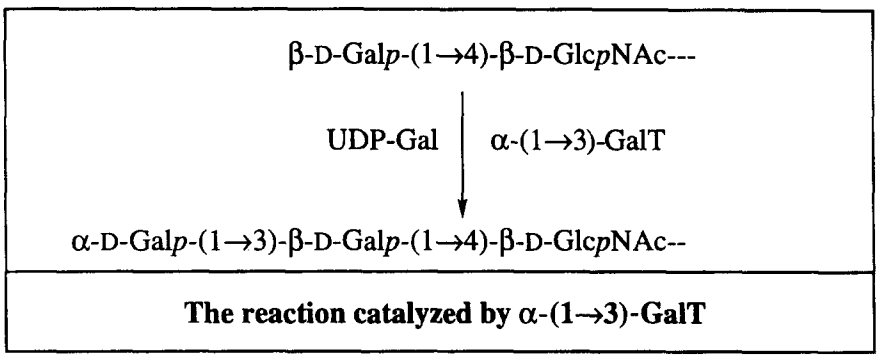
(1)

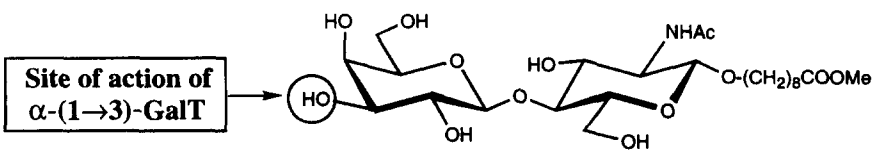
are important targets for synthetic carbohydrate chemistry since such compounds could be of tremendous value in the study of both oligosaccharide biosynthesis [5,6] and function [7].

To date, only limited success has been reported in the design and synthesis of such inhibitors having sub-millimolar inhibition constants. Masking of the acceptor hydroxyl group to which an enzyme transfers, by either deoxygenation [5,8–12], *O*-methylation [6,13] or replacement by thio-groups [10,11,14] has met with some success as has *O*-methylation at an adjacent position [6,15,16]. The most potent (sub-micromolar) inhibitor reported to date, however, was obtained by replacing the hydroxyl group (OH-3) of the Gal-residue undergoing glycosylation with an amino group in the disaccharide, α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-OR, a substrate for the human blood group-A encoded α-(1 → 3)-*N*-acetylgalactosaminyltransferase [13]. In this glycosylation reaction, the sugar is transferred from the sugar nucleotide with retention of configuration, unlike in previous systematic studies [5,6] where the glycosyltransferases examined acted mechanistically with inversion of configuration. An amino-inhibitor with K_i near 200 nM was obtained [13] for the *N*-acetylgalactosaminyltransferase proceeding with retention of configuration; however, the mode of inhibition was complex [17] and not purely competitive as had been expected.

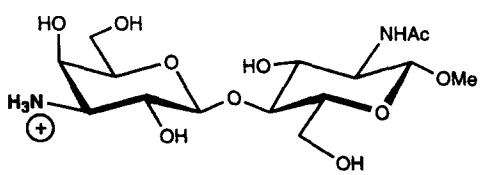
The objective of the present study was to assess whether a similar strategy, that of replacing the reactive hydroxyl group of the acceptor by an amino group, would produce an inhibitory analog for α-(1 → 3)-D-galactosyltransferase (GalT), which also transfers a glycosyl residue with retention of configuration from the sugar nucleotide. This enzyme transfers galactose from UDP-D-galactose to OH-3 of the Gal residue in oligosaccharide sequences terminating in β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc (*N*-acetyllactosamine, LacNAc). Considerable interest has developed in the regulated expression of this α-(1 → 3)-GalT since the discovery of a naturally occurring human antibody (anti-Gal) to glycoconjugates with a non-reducing terminal α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-OR epitope [18,19]. It has previously been demonstrated that the expression

of α-(1 → 3)-GalT occurs in a species-specific manner and accounts for the evolutionarily restricted expression of α-D-Gal *p*-(1 → 3)-β-D-Gal *p*-(1 → 4)-β-D-Glc *p*NAc-terminated glycoconjugates [20–22]. These discoveries have had a direct impact on both the development of therapeutic glycoproteins by recombinant methods and the use of animal tissues for xenotransplantation [23,24]. Therefore, the development of a specific inhibitor of α-(1 → 3)-GalT may be of therapeutic value.





Synthetic acceptor **10** (K_m = 190 μM)



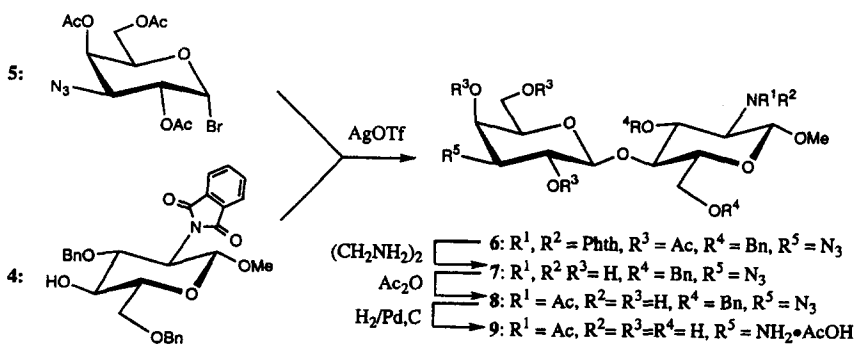
Potential inhibitor **9**

2. Results and discussion

The target potential inhibitor methyl 3-amino-3-deoxy-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranoside (**9**) was synthesized as follows (Scheme 1). Reaction of known [25] methyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**4**) with the previously reported [13] 3-azido galactosyl donor **5** gave the expected β-linked protected disaccharide **6** in 82% yield. Ethylenediamine [26] was used for removal of the phthalimido protecting group to produce the free amine (**7**) that was *N*-acetylated to give **8** (2 steps, 94%). Hydrogenolysis in acetic acid gave the required **9** as the acetate salt, which was characterized by NMR spectroscopy and mass spectrometry ($M + H^+ = 397$). Compound **9** was further confirmed to be the acetate salt, and not the 3′-*N*-acetyl derivative, since lyophilization from an aqueous solution containing added CD_3CO_2D yielded a residue where the protons of the methyl group of the acetate counter ion had disappeared from the ^1H NMR spectrum.

Amino-disaccharide **9** was tested as an inhibitor of the recombinant α-(1 → 3)-GalT [27] in the transfer of galactose to β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-O$(CH_2)_8$COOMe (**10**) [5], which, for this acceptor, was found to have $K_m = 190 \pm 6$ μM and $V_{max} = 0.92$ nmol/h under standard conditions (see Experimental section). The hydrophobic aglycon present in acceptor **10** allowed the use of the so-called [28] "Sep-Pak assays" to determine the kinetic parameters for the transfer of tracer-[^{3}H]-labeled galactose from UDP-galactose to OH-3′ of the Gal*p*-residue in **10**. The effect of added amino-disaccharide (**9**) on the rate of transfer is shown in Fig. 1. Clearly, the mode of inhibition of **9** is not competitive. Within experimental error, the apparent K_m for acceptor was unchanged by the addition of **9**. V_{max}, however, decreased with increasing inhibitor concentration; a pattern that is consistent with non-competitive inhibition. Analysis [29] of the data in Fig. 1 yields $K_i = 104\mu M$, almost half that of the acceptor K_m.

It seemed surprising that **9** is not a competitive inhibitor, which would be expected if it bound to the enzyme purely as an acceptor analog. The β-octyl glycoside of 3-amino-3-deoxy-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose [30] was not a substrate when evaluated in the Sep-Pak assay, so it is unlikely that **9** underwent galactosylation to complicate the kinetic analysis. Despite the non-competi-



Scheme 1.

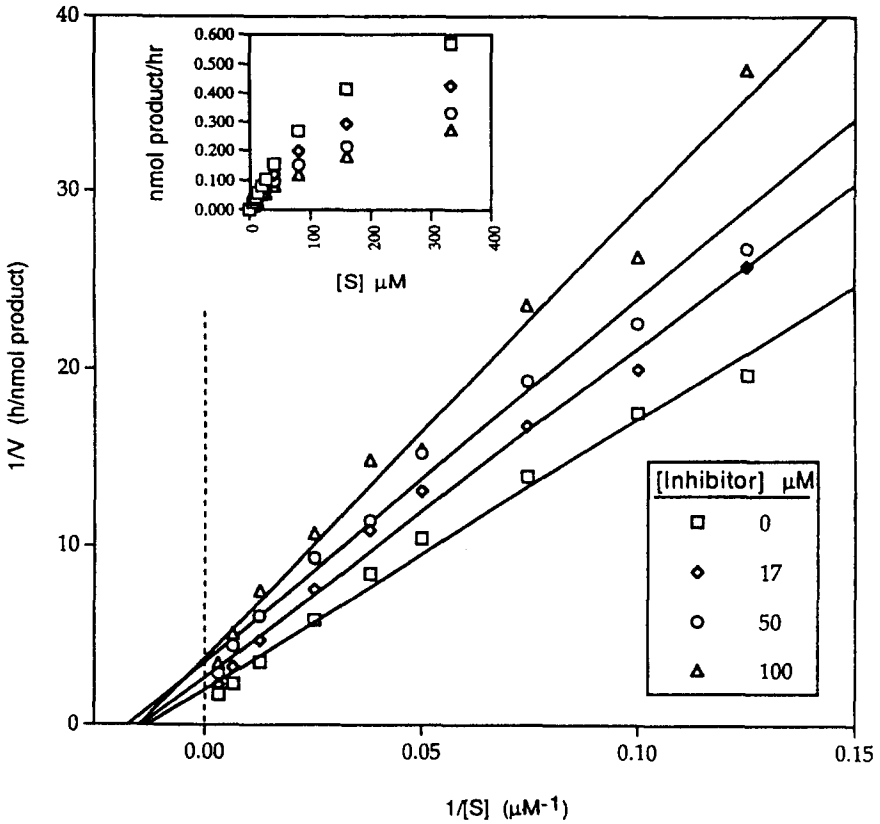


Fig. 1. Kinetic analysis of the inhibition of α-(1 → 3)-GalT by amino-disaccharide **9**. The Lineweaver–Burk plot of the inhibition by **9** was generated using KinetAssyst II from the Michaelis–Menten plot shown in the inset [29]. Substrate **10** concentration was varied from 6–332 μM. Variations in the concentrations of **9** are as shown.

tive behavior, the inhibition appears to be specific, since no inhibition was observed when **9** was evaluated as a potential inhibitor for either bovine β-(1 → 4)-galactosyltransferase [5] (which utilizes UDP-Gal as a donor, but proceeds with inversion of configuration) or an α-(1 → 4)-galactosyltransferase [31] (which utilizes UDP-Gal as the donor and proceeds with retention of configuration). Also, compound **9** did not inhibit α-(1 → 3/4)-fucosyltransferase from human milk (which utilizes the acceptor **10**, but transfers fucose from GDP-fucose to OH-3 of the GlcNAc residue). The inhibition therefore does not appear to depend on recognition of the donor sugar nucleotide nor some non-specific property of amino-disaccharide **9**.

It should be noted that, while non-competitive inhibition is often interpreted as the result of an inhibitor binding to a site independent of the substrate site, this mode of inhibition is commonly observed in multisubstrate enzyme reactions where dead-end inhibitor complexes can be formed [32,33]. If a rate-determining step occurs after the formation of the ES complex, a non-competitive inhibitory pattern will be observed.

This has been clearly demonstrated for aldose reductase where crystallographic studies show non-competitive and uncompetitive inhibitors in substrate binding sites [34–36]. The absence of detailed mechanistic characterizations of the α-galactosyltransferase reaction, which are beyond the scope of the current study, precludes a clear interpretation of the inhibitor results. What should be emphasized, however, is that this is the first inhibitor reported for this enzyme, the inhibition is specific, and the K_i is significantly lower than the K_m for the corresponding acceptor.

3. Experimental

General methods.—Optical rotations were measured with a Perkin–Elmer 241 polarimeter at 22 ± 2°C. Analytical TLC was performed on Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by quenching of fluorescence and/or by charring with sulfuric acid. All commercial reagents were used as supplied, and chromatography solvents were distilled prior to use. Column chromatography was performed on Silica Gel 60 (40–60 μM, E. Merck, Darmstadt). ^{1}H NMR spectra were recorded at 360 MHz (Bruker WM-360) with either internal $(CH_3)_4Si$ (δ 0, $CDCl_3$, CD_3OD) or DOH (δ 4.80, D_2O). ^{13}C NMR spectra were recorded at 75.5 MHz (Bruker AM-300) with internal $(CH_3)_4Si$ (δ 0, $CDCl_3$, CD_3OD) or external 1,4-dioxane (δ 67.4, D_2O). ^{1}H NMR data are reported as though they were first order. Unless otherwise stated, all reactions were carried out at room temperature, and, in the processing of reaction mixtures, solutions of organic solvents were washed with equal amounts of aqueous solutions. Organic solutions were dried (magnesium sulfate) prior to concentration under vacuum at < 40°C (bath). Microanalyses were carried out by the analytical services at this department, and all samples submitted for elemental analyses were dried overnight under vacuum with phosphorous pentoxide at 56°C (refluxing acetone). Mass spectra were recorded on samples suspended in a Cleland's matrix (1:5 1,4-dithiothreitol-1,4-dithioerythritol) using a Kratos AEIMS9 instrument with xenon as the bombarding gas.

Methyl 2,4,6-tri-O-acetyl-3-azido-3-deoxy-β-D-galactopyranosyl-(1 → 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**6**).—Silver triflate (350 mg, 1.4 mmol) was added at −40°C to a stirred mixture of methyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside [25] (**4**) (294 mg, 0.58 mmol), and 2,4,6-tri-*O*-acetyl-3-azido-3-deoxy-β-D-galactopyranosyl bromide [13] (**5**) (390 mg, 0.9 mmol) in dichloromethane (20 mL) containing ground molecular sieves (4 Å) and maintained under nitrogen. After 1 h, TLC (1:1 hexane–ethyl acetate) indicated the reaction to be complete, and 10% sodium thiosulfate was added. The mixture was allowed to attain room temperature, and then it was filtered through Celite. The organic layer was separated, washed with water, dried, filtered, and concentrated. Column chromatography (2:1 hexane–ethyl acetate) gave **6** (389 mg, 0.48 mmol, 82%): $[\alpha]_D$ + 27° (*c* 0.77, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 3.20 (dd, 1 H, $J_{2',3'}$ 10.6, $J_{3',4'}$ 3.4 Hz, H-3'), 3.53 (1 H, H-5), 3.60 (1 H, H-5'), 3.82 (2 H, H-6), 3.92 (2 H, H-6'), 4.05 (1 H, H-4), 4.13 (dd, 1 H, $J_{1,2}$ 8.4, $J_{2,3}$ 10.7 Hz, H-2), 4.23 (dd, 1 H, $J_{3,4}$ 8.5 Hz, H-3), 4.52 (d, 1 H, $J_{1',2'}$ 8.0 Hz, H-1'), 5.02 (d, 1 H, $J_{1,2}$ 8.4 Hz, H-1), 5.07 (dd, 1 H, H-2'), 5.27 (1 H, H-4'); ^{13}C NMR:

δ 20.5, 20.7, 20.8 (Ac CH_3), 55.5, 56.7 (OCH_3, C-2), 60.9–79.2 (C-3,4,5,6, C-2′,3′,4′,5′,6′, PhCH_2), 99.3, 100.5 (C-1, C-1′), 123.2–138.5 (ArC), 169.0, 169.9, 170.3 (Ac C = O). Anal. Calcd for $C_{41}H_{44}N_4O_{14}$: C, 60.29; H, 5.43; N, 6.86. Found: C, 60.34; H, 5.41; N, 6.89.

*Methyl 3-azido-3-deoxy-β-*D*-galactopyranosyl-(1 → 4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-*D*-glucopyranoside* (**8**).—Ethylenediamine [26] (3 mL) was added to **6** (288 mg, 0.35 mmol) in *n*-butanol (15 mL). The mixture was stirred at 70°C overnight, concentrated and coevaporated twice with toluene. The residue (**7**) was dissolved in methanol (5 mL) and treated with acetic anhydride (1 mL) at room temperature. After 2 h the reaction mixture was concentrated. Column chromatography (9:1 ethyl acetate–methanol) gave **8** (200 mg, 0.33 mmol, 94%); $[\alpha]_D$ − 8° (*c* 1.6, CH_3OH); ^{1}H NMR (CD_3OD): δ 1.90 (s, 3 H, AcNH CH_3), 3.15 (dd, 1 H, $J_{2',3'}$ 10.4, $J_{3',4'}$ 3.2 Hz, H-3′), 3.45 (s, 3 H, OCH_3), 3.65 (1 H, H-3), 3.85 (1 H, H-4′),3.79 (1 H, H-2), 3.75 (1 H, H-2′), 4.36 (d, 1 H, $J_{1,2}$ 8.4 Hz, H-1), 4.42 (d, 1 H, $J_{1',2'}$ 7.6 Hz, H-1′); ^{13}C NMR: δ 23.0 (AcNH CH_3), 56.2, 57.0 (OCH_3, C-2), 62.8–82.4 (C-3,4,5,6, C-2′,3′,4′,5′,6′, PhCH_2), 103.5, 104.4 (C-1, C-1′), 128.7–139.7 (ArC), 173.2 (AcNH C = O).

*Methyl 3-amino-3-deoxy-β-*D*-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-*D*-glucopyranoside, acetate salt* (**9**).—Compound **8** (153 mg, 0.25 mmol) was hydrogenolyzed in acetic acid (1 mL) over 10% Pd/C at atmospheric pressure for 5 h. The mixture was filtered through Celite and concentrated. Column chromatography on Iatrobeads (6:3:3:2 ethyl acetate–methanol–acetic acid–water) gave amorphous **9** (91 mg, 0.20 mmol, 79%) after evaporation and lyophilization. ^{1}H NMR (D_2O): δ 1.90, 2.02 (2 s, 6 H, AcNH, Ac CH_3), 3.47 (dd, 1 H,$J_{2',3'}$ 10.8, $J_{3',4'}$ 3.2 Hz, H-3′), 3.51 (s, 3 H, OCH_3), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.58 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′); ^{13}C NMR: δ 22.5, 23.0 (AcNH, AcCH_3), 55.9–79.2 (OCH_3, C-2,3,4,5,6, C-2′,3′,4′,5′,6′), 102.7, 103.5 (C-1, C-1′), 175.5 (AcNH C=O), 179.6 (Ac C=O). FABMS *m/z* 397 $(M + H)^+$ and 419 $(M + Na)^+$.

Measurement of enzyme kinetics.—The enzyme, α-(1 → 3)-GalT, was prepared as described by Henion et al. [27] using a coding sequence for the murine form of the enzyme [37]. Kinetic evaluations were done by varying the concentrations of acceptor **10** from 6–332 μM in the absence and presence of inhibitor **9** (17, 50 and 100 μM). The kinetic parameters, K_m and V_{max}, for each set of conditions were determined using the Kinet Assyst program to fit the velocity vs acceptor **10** data to the Michaelis–Menten equation [29].

Acknowledgements

We thank Ms C. Compston for performing preliminary enzyme assays. This work was supported by the Medical Research Council of Canada through grants to both M.M.P and O.H. B.A.M was supported by the National Institutes of Health (GM 40205). Dr F.A. Quiocho is acknowledged for invaluable discussion on potential interpretations of non-competitive inhibition.

References

[1] T.A. Beyer, J.E. Sadler, J.I. Rearick, J.C. Paulson, and R.L. Hill, *Adv. Enzymol.*, 52 (1981) 23–175.
[2] H. Schachter, *Biochem. Cell Biol.*, 64 (1986) 163–181.
[3] H. Schachter, *Current Opin. Struct. Biol.*, 1 (1991) 755–765.
[4] D.H. van den Eijnden and D.H. Joziasse, *Current Opin. Struct. Biol.*, 3 (1993) 711–721.
[5] O. Hindsgaul, K.J. Kaur, G. Srivastava, M. Blaszczyk-Thurin, S.C. Crawley, L.D. Heerze, and M.M. Palcic, *J. Biol. Chem.*, 266 (1991) 17858–17862.
[6] S.H. Khan and K.L. Matta, in H.J. Allen and E.C. Kiselius, (Eds)., *Glycoconjugates: Composition, Structure, and Function*, Marcel Dekker, New York, 1992, pp 361–378.
[7] A. Varki, *Glycobiology*, 3 (1993) 97–130.
[8] M.M. Palcic, J. Ripka, K.J. Kaur, M. Shoreibah, O. Hindsgaul, and M. Pierce, *J. Biol. Chem.*, 265 (1990) 6759–6769.
[9] W. Kuhns, V. Rutz, H. Paulsen, K.L. Matta, M.A. Baker, M. Barner, M. Granovsky, and I. Brockhausen, *Glycoconjugate. J.*, 10 (1993) 381–394.
[10] Y. Kajihara, H. Hashimoto, H. Kodama, T. Wakabayshi, and K. Sato, *J. Carbohydr. Chem.*, 12 (1993) 991–995.
[11] Y. Kajihara, H. Kodama, T. Wakabayshi, K. Sato, and H. Hashimoto, *Carbohydr. Res.*, 247 (1993) 179–193.
[12] T.L. Lowary and O. Hindsgaul, *Carbohydr. Res.*, 249 (1993) 163–195.
[13] T.L. Lowary and O. Hindsgaul, *Carbohydr. Res.*, 251 (1994) 33–67.
[14] Y. Kajihara, H. Hashimoto, and H. Kodama, *Carbohydr. Res.*, 229 (1992) C5–C9.
[15] G. Möller, F. Reck, H. Paulsen, K.J. Kaur, M. Sarkar, H. Schachter, and I. Brockhausen, *Glycoconjugate J.*, 9 (1992) 180–190.
[16] S.H. Khan, S.C. Crawley, O. Kanie, and O. Hindsgaul, *J. Biol. Chem.*, 268 (1993) 2468–2473.
[17] T.L. Lowary, Ph.D. Thesis, University of Alberta, 1993.
[18] U. Galili, E.A. Rachmilewitz, A. Peleg, and I. Flechner. *J. Exp. Med.*, 160 (1984) 1519–1531.
[19] U. Galili, J. Buehler, S.B. Shohet, and B.A. Macher, *J. Exp. Med.*, 162 (1985) 573–582.
[20] U. Galili, M.R. Clark, S.B. Shohet, J. Buehler, and B.A. Macher, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 2092–2096.
[21] U. Galili, S.B. Shohet, E. Kobrin, C.L.M. Stults, and B.A. Macher, *J. Biol. Chem.*, 263, (1988) 17755–17762.
[22] U. Galili and K. Swanson, *Proc. Natl. Acad. Sci. USA*, 88 (1991) 7401–7404.
[23] U. Galili, *Lancet, 2* (1989) 358–361.
[24] A.H. Good, D.K.C. Cooper, A.J. Malcolm, R.M. Ippolito, F.A. Neethling, Y. Ye, N. Zuhdi, and L.R. Lamontagne, *Transplant. Proc.*, 24 (1992) 559–562.
[25] D.A. Schwartz, H.-H. Lee, J.P. Carver, and J.J. Krepinsky, *Can. J. Chem.*, 63 (1985) 1073–1079.
[26] O. Kanie, S.C. Crawley, M.M. Palcic, and O. Hindsgaul, *Carbohydr. Res.*, 243 (1993) 139–164.
[27] T.R. Henion, B.A. Macher, F. Anaraki, and U. Galili, *Glycobiology*, 4 (1994) 193–201.
[28] M.M. Palcic, L.D. Heerze, M. Pierce, and O. Hindsgaul, *Glycoconjugate J.*, 5 (1988) 49–63.
[29] C.L.M. Stults and B.A. Macher, *Arch. Biochem. Biophys.*, 303 (1993) 125–133.
[30] C.L.M. Stults, R.D. Larsen, and B.A. Macher, *Glycoconjugate J.*, in press.
[31] A.C. Helland, O. Hindsgaul, and M.M. Palcic. *Abstr. Papers, XVIIth Intl. Carbohydr. Symp.*, (1994) 225, Abstr. B1.63.
[32] W.W. Cleland, *The Enzymes*, 2 (1970) 1–65.
[33] J.A. Todhunter, *Methods Enzymol.*, 63 (1979) 383–481.
[34] I. Tarle, D.W. Borhan, D.K. Wilson, F.A. Quicho, and J.M. Petrash, *J. Biol. Chem.*, 268 (1993) 25687–25693.
[35] D.K. Wilson, I. Tarle, J.M. Petrash, and F.A. Quicho, *Proc. Natl. Acad. Sci. USA.*, 90 (1993) 9847–9851.
[36] J.M. Petrash, I. Tarle, D.K. Wilson, and F.A. Quicho, *Pers. Diab.*, 43 (1994) 955–959.
[37] R.D. Larsen, V.P. Rajan, M. Ruff, J. Kukowska-Lattallo, R.D. Cummings, and J. Lowe, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 8227–8231.