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UDP-Gal: GlcNAc-R β1,4-galactosyltransferase—a target enzyme for drug design. Acceptor specificity and inhibition of the enzyme

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Abstract Galactosyltransferases are important enzymes for the extension of the glycan chains of glycoproteins and glycolipids, and play critical roles in cell surface functions and in the immune system. In this work, the acceptor specificity and several inhibitors of bovine β1,4-Gal-transferase T1 (β4GalT, EC 2.4.1.90) were studied. Series of analogs of *N*-acetylglucosamine (GlcNAc) and GlcNAc-carrying glycopeptides were synthesized as acceptor substrates. Modifications were made at the 3-, 4- and 6-positions of the sugar ring of the acceptor, in the nature of the glycosidic linkage, in the aglycone moiety and in the 2-acetamido group. The acceptor specificity studies showed that the 4-hydroxyl group of the sugar ring was essential for β4GalT activity, but that the 3-hydroxyl could be replaced by an electronegative group. Compounds having the anomeric β-configuration were more active than those having the α-configuration, and *O*-, *S*- and *C*-glycosyl compounds were all active as substrates. The aglycone was a major determinant for the rate of Gal-transfer. Derivatives containing a 2-naphthyl aglycone were inactive as substrates although quinolinyl groups supported activity. Several compounds having a bicyclic structure as the aglycone were found to bind to the enzyme and inhibited the transfer of Gal to control substrates. The best small hydrophobic GlcNAc-analog inhibitor was found to be 1-thio-*N*-butyrylGlcNβ-(2-naphthyl) with a K_i of 0.01 mM. These studies help to delineate β4GalT–substrate interactions and will aid in the development of biologically applicable inhibitors of the enzyme.

Keywords Galactosyltransferase · Substrate specificity · Substrate analogs · Enzyme kinetics · Inhibitors

Abbreviations

Bn	benzyl
β4GalT	β1,4-galactosyltransferase
Me	methyl
Ph	phenyl
rt	room temperature

Introduction

In mammals, the extension of glycoconjugate oligosaccharide chains by galactose (Gal) plays a critical role in mediating a variety of biological recognition processes such as cell adhesion, microbial adhesion, fertilization, apoptosis, leukocyte adhesion to the endothelium and inflammation. β1,4-Gal-transferases (β4GalT) are involved in the synthesis of galactosylated recognition determinants such as sialyl-Lewis^x which are ligands for selectins and are important for leukocyte adhesion to the endothelium [1,2]. β4GalT belongs to the CAZY family 7 of inverting glycosyltransferases (http://afmb.cnrs-mrs.fr/CAZY/) which catalyze the transfer of Gal from UDP-Gal to GlcNAc-terminating acceptors with inversion of configuration of the glycosidic linkage. In the presence of α-lactalbumin, the enzyme changes its specificity to become lactose synthase, and transfers Gal to glucose to synthesize lactose [3]. The terminal GlcNAc residues that serve as galactosyl acceptors are usually components of

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more-complex underlying oligosaccharide structures which can also have a potential effect in regulating the rates of galactosyl transfer due to an extended binding site of the enzyme. The serum or tissue levels of β4GalT appear to be transcriptionally or translationally controlled and, for example, have been found to increase in specific cancers [4–6]. We have shown that inflammatory conditions are also associated with increased β4GalT activity [7]. Thus, inhibition of galactosylation should alter the concentrations of important adhesion determinants and could be utilized as a potential treatment for a number of disease conditions.

The human β4GalT family consists of enzyme proteins that share sequence similarity yet differ in tissue expression and possibly in acceptor substrate specificity [8]. X-ray crystallography of the soluble domain of bovine β4GalT1 suggests that the enzyme binds Mn^{2+} and its donor substrate UDP-Gal inside a deep catalytic pocket [9]. The binding of UDP-Gal is thought to induce a conformational change in the enzyme that creates the acceptor binding site [10]. The extended pocket that binds GlcNAc-terminating substrates contains a number of hydrophobic amino acids including Phe, Ile, Tyr and Trp [11,12]. With oligosaccharide substrates having several terminal non-reducing GlcNAc residues, the enzyme exhibits a definite branch specificity [13].

Certain nucleoside methylenediphosphonate sugars [14] and a complex UDP-sugar resembling the proposed transition state of glycosyl transfer [15] have been found to be effective β4GalT inhibitors. Synthetic 3- and 4-deoxyGlcNAc derivatives were shown to be inactive as substrates, and these compounds either did not inhibit or poorly inhibited the enzyme, indicating that these compounds did not bind well to the enzyme [16,17]. However, 4-amino-4-deoxyGlcNAc significantly inhibited the enzyme [18], suggesting that hydrogen bonding to the 4-substituent of Gal may be important for substrate binding to the enzyme. A number of acceptors containing modifications at the 6-position of GlcNAc were good substrates, including 6-azido-6-deoxyGlcNAc and 6-deoxy-6-fluoroGlcNAc. By comparison, 6-deoxy-6-aminoGlcNAc-R, 6-*O*-acetylGlcNAc-R [19] and 6-thioGlcNAc-R were poor substrates [18,20,21]. The presence of sialic acid at the 6-position of GlcNAc was not favourable while fucose in the 6-position was accepted by the enzyme. The oxygen atom in the ring of the GlcNAc moiety in the substrate could be replaced by carbon [22]. GlcNAc derivatives having 2-naphthyl aglycone groups in the β-configuration were reported to be inactive as substrates; however, some of these derivatives strongly inhibited the enzyme [23].

Here we report the results of an investigation on series of compounds designed to extend the knowledge of the acceptor-substrate specificity of bovine milk β4GalT (EC 2.4.1.90), and in particular, to study the role of underlying structures in acceptor-substrate binding. The information obtained should be useful for an understanding of enzyme recognition of the substrate, and for the design of biologically applicable galactosylation inhibitors that are small and have hydrophobic groups facilitating the penetration into cells.

Materials and methods

Materials

The synthesis of the naphthyl glycosides **11** and **15**, the naphthyl thioglycosides **9**, **10** and **12**, the isoquinolinyl glycoside **24**, the quinolinyl glycosides **25**–**28**, and the 6-thio glycoside **29** is described below. Other compounds were either purchased or obtained as follows. Compounds **1**, **2**, **4**, **5**, **20** were purchased from Sigma Chemical Co., St. Louis, MO. Glycopeptides **GP1**–**GP13** were obtained as described by Mathieux et al. [24]. The oligosacharides **21**–**23** were obtained from Dr. Khushi Matta, Roswell Park Cancer Institute, Buffalo, NY. The *C*-glycosyl compound **6** and the 1-deoxy aza sugar **42** were provided by Dr. Konrad Sandhoff, Bonn University, Bonn, Germany. The calystegines **39**–**41** were obtained from Dr. Robert Madsen, Technical University of Denmark, Lyngby, Denmark. The remaining GlcNAc and other *N*-substituted GlcN derivatives were obtained as described in the corresponding references: **3** [25], **7** [26], **8** [27,28], **13** [29], **14** [26], **16** [30], **17** [31], **30** [32], **31** [33], **32** [34], **33** [35], **34** and **35** [36], **36** [37], **37** [38], **38** [39], **44** [40], **45** [41], **46** [34], **47**–**50** [38]. The synthesis of the α- and β-anomers (**18** and **19**) of GlcNAc-(11-phenoxyundecyl) will be described elsewhere. 2-Butanamido-2-deoxy-D-glucopyranose was prepared by the procedure of Ponticelli et al. [42]. The remaining materials required for biological assays were obtained as described [43] or were purchased from Sigma Chemical Co, St. Louis, MO. Galactosyltransferase from bovine milk (EC 2.4.1.22 or EC 2.4.1.90) was purchased from Sigma (6.8 U/mg protein).

Synthesis of substrates and inhibitors

The synthesis of the naphthyl glycosides **11** and **15**, the naphthyl thioglycosides **9**, **10** and **12**, the isoquinolinyl glycoside **24**, and the quinolinyl glycosides **25**–**28** is shown in Scheme 1. The approach is based on the phase-transfer catalytic methodology described by Roy and Tropper [28]. The method involves treatment of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) [44] or 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-α-D-glucopyranosyl chloride (**52**)[42] with the appropriate naphthol, thionaphthol, quinolinol, thioquinolinol, or isoquinolinol in the presence of tetra-*n*-butylammonium bromide in the two-phase system of methylene chloride and an aqueous solution of sodium hydroxide or, tetra-*n*-butylammonium hydrogensulfate in the two-phase system of ethyl acetate and an aqueous solution of sodium carbonate. De-*O*-acetylation using

sodium methoxide in methanol afforded the target glycoside or thioglycoside. The synthesis of the 6-thio derivatives of the benzyl glycoside **29**, the 2-naphthyl glycoside **15**, and the 2-naphthyl thioglycoside **12** is shown in Scheme 2. The method involves the selective tosylation of the primary hydroxyl in the corresponding starting glycoside **1**, **11** or **10**, followed by the acetylation of the remaining hydroxyl groups, and subsequent displacement of the tosyloxy group using potassium thioacetate. Alkaline deacetylation afforded the target glycosides.

The *N*-butyryl compounds were prepared from 2-butanamido-2-deoxy-D-glucopyranose which was obtained from D-glucosamine by treatment with butanoic anhydride, following the procedure of Ponticelli et al. [42]. *N*-ButrylGlcNβ-Bn (**43**) was then prepared by the selective alkylation of 2-butanamido-2-deoxy-D-glucopyranose using benzyl bromide, following the procedure by Vauzeilles et al. [30].

General methods

The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300-, 400- and 500-MHz spectrometers in $CDCl_3$, CD_3OD, or DMSO-d_6. The signals owing to residual protons in the deuterated solvents were used as internal standards. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane [45]. Carbon chemical shifts are given relative to $CDCl_3$, CD_3OD or DMSO-d_6: $\delta = 77.16$, 49.00 or 39.52 ppm, respectively. For simplicity, only characteristic features of the ^{1}H and ^{13}C spectra are reported. High-resolution electrospray mass spectra were recorded on an Applied Biosystems/MDS Sciex QSTAR XL spectrometer with an Agilent HP1100 Cap-LC system. Samples were run in 50% aqueous MeOH at a flow rate of 6μL/min . Melting points were determined on a Mel-Temp II melting point apparatus and are uncorrected. Optical rotations were measured using an Autopol® II automatic polarimeter for solutions in a 1-dm cell at rt. Thin-layer chromatography was performed using glass- or aluminum-backed Silicyle Silica Gel 60 F_{254} plates. Plates were viewed by charring after spraying with either 5% H_2SO_4 in EtOH, or phosphomolybdic acid (PMA) in EtOH.

Representative procedure for the synthesis of naphthyl, quinolinyl and isoquinolinyl glycosides and thioglycosides

An aqueous solution of 1 M sodium hydroxide (3 mL) was added to a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) or 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-α-D-glucopyranosyl chloride (**52**) (1.1 mmol), aryl alcohol or thiol (2.2 mmol, 2 equiv), and tetra-*n*-butylammonium bromide (1.1 mmol, 1 equiv) in methylene chloride (4 mL). The resulting two-phase system was stirred for 2 h at rt. The mixture was diluted with ethyl acetate (50 mL) and the organic phase washed sequentially with an aqueous solution of 1 M sodium hydroxide (40 mL) and water (2 × 20 mL), and dried (Na_2SO_4). The organic extract was then filtered and the filtrate concentrated to yield a crude solid which was recrystallized from ethyl acetate–hexanes to give the target aryl glycosides or thioglycosides.

Representative procedures for the de-*O*-acetylation of aryl glycosides or thioglycosides

Two methods were used for the de-*O*-acetylation of aryl glycosides or thioglycosides.

Method A

To a solution of the acetylated substrate in methanol was added a catalytic amount of 1 M sodium methoxide in methanol. The reaction mixture was stirred at rt and the progress of the reaction was monitored by TLC {4:1 (v/v), $CHCl_3$–MeOH}. Upon completion, Amberlite IR-120 (H^+) ion-exchange resin was added, and the mixture was stirred for 20 min. The suspension was then filtered and the filtrate concentrated to give the de-*O*-acetylated product.

Method B

To a solution of the acetylated substrate in methanol–toluene {1:1 (v/v)} was added a catalytic amount of 0.5 M sodium methoxide in methanol. The reaction mixture was stirred at rt and the progress of the reaction was monitored by TLC {4:1 (v/v), $CHCl_3$–MeOH}. Upon completion, an equal volume of hexane was added, the mixture was cooled, and the precipitated product was collected by filtration.

2-Naphthyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside

To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) (1.04 g, 2.85 mmol), 2-naphthalenethiol (0.91 g, 5.69 mmol), and tetra-*n*-butylammonium hydrogensulfate (0.97 g, 2.04 mmol) in ethyl acetate (10 mL) was added an aqueous 1.5 M solution of sodium carbonate (10 mL). The resulting two-phase system was then stirred for 1.5 h at rt. The mixture was then diluted with ethyl acetate (75 mL) and the organic phase washed sequentially with a solution of saturated sodium hydrogencarbonate (2 × 50 mL), water (2 × 50 mL), and brine (50 mL), and dried (Na_2SO_4). The organic extract was filtered and the filtrate was concentrated to yield a crude solid which was recrystallized (ethyl acetate–hexanes) to give the title compound (0.67 g, 44%) as a white solid: mp 212–214°C; $[\alpha]_D$ –18.2° (*c* 1.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$):

δ 1.98, 1.99, 2.00, and 2.01 (4 s, 12 H, 3 OAc, NAc), 4.95 (d, 1 H, *J* 10.4 Hz, H-1), 7.43–7.97 (7 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.5, 20.7, and 20.7 (3 OCO*C*H_3), 23.3 (NCO*C*H_3), 86.6 (C-1), 126.5, 126.6, 127.6, 127.7, 128.4, 129.7, 129.8, 131.7, 132.7, and 133.5 (Ar), 169.3, 170.1, 170.6, and 171.0 (3 O*C*OCH$_3$, N*C*OCH$_3$); HRMS (ES): Calcd for $C_{24}H_{27}NO_8SNa$ ([M + Na]$^+$): 512.1355. Found: 512.1344.

2-Naphthyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside (**9**)

A sample (0.35 g, 0.72 mmol) of 2-naphthyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside prepared as described above was de-*O*-acetylated following Method B to give **9** (0.16 g, 63%) as a white solid: mp 241–242°C (dec); $[\alpha]_D$ +36.4° (*c* 0.1, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.84 (s, 3 H, NAc), 4.86 (d, 1 H, *J* 10.5 Hz, H-1), 7.45–7.98 (7 H, Ar); ^{13}C NMR (126 MHz, DMSO-d_6): δ 23.1 (NCO*C*H_3), 86.2 (C-1), 125.8, 126.6, 126.9, 127.2, 127.3, 127.6, 128.1, 131.4, 133.3, and 133.4 (Ar), 169.2 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{18}H_{21}NO_5SNa$ ([M + Na]$^+$): 386.1038. Found: 386.1038.

2-Naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside

To a solution of 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-α-D-glucopyranosyl chloride (**52**) (0.67 g, 1.70 mmol), 2-naphthalenethiol (0.55 g, 3.41 mmol), and tetra-*n*-butylammonium hydrogensulfate (0.58 g, 1.70 mmol) in ethyl acetate (10 mL) was added an aqueous 1.5 M solution of sodium carbonate (10 mL). The resulting two-phase system was then stirred for 1.5 h at rt. The mixture was then diluted with ethyl acetate (75 mL) and the organic phase washed sequentially with a solution of saturated sodium hydrogencarbonate (2 × 50 mL), water (2 × 50 mL), and brine (50 mL), and dried (Na_2SO_4). The organic extract was filtered and the filtrate was concentrated to yield a crude solid which was recrystallized (ethyl acetate–hexanes) to give the title compound (0.66 g, 75%) as a white solid: mp 179–181°C; $[\alpha]_D$–14.2° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.99 (t, 3 H, *J* 7.4 Hz, C*H*$_3$CH$_2$), 1.70 (m, 2 H, CH$_3$C*H*$_2$), 2.03, 2.04, and 2.05 (3 s, 9 H, 3 OAc), 2.19 (t, 2 H, *J* 7.0 Hz, C*H*$_2$CO), 4.97 (d, 1 H, *J* 10.4 Hz, H-1), 7.50–7.88 (7 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.7 (*C*H$_3$CH$_2$), 19.0 (CH$_3$*C*H$_2$), 20.6 and 20.7 (2 C) (3 OCO*C*H$_3$), 38.7 (*C*H$_2$CO), 88.5 (C-1); HRMS (ES): Calcd for $C_{26}H_{32}NO_8S$ ([M + H]$^+$): 518.1849. Found: 518.1841.

2-Naphthyl 2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside (**10**)

A sample (0.50 g, 0.98 mmol) of 2-naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside prepared above was de-*O*-acetylated following Method B to give **10** (0.37 g, 97%) as a white solid: mp 212–214°C (dec); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.91 (t, 3 H, *J* 7.4 Hz, C*H*$_3$CH$_2$), 1.56 (m, 2 H, CH$_3$C*H*$_2$), 2.08 (t, 2 H, *J* 7.0 Hz, C*H*$_2$CO), 4.88 (d, 1 H, *J* 10.4 Hz, H-1), 7.46–7.88 (7 H, Ar); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.7 (*C*H$_3$CH$_2$), 18.7 (CH$_3$*C*H$_2$), 37.9 (*C*H$_2$CO), 86.6 (C-1), 125.9, 126.6, 127.0, 127.3 (2 C), 127.6, 128.2, 131.4, and 133.5 (2 C) (Ar), 172.2 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{20}H_{26}NO_5S$ ([M + H]$^+$): 392.1532. Found: 392.1533.

2-Naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-β-D-glucopyranoside

The title compound was prepared from 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-α-D-glucopyranosyl chloride (**52**) (0.32 g, 0.80 mmol) and 2-naphthol following the representative procedure for the synthesis of aryl glycosides and was obtained as a white solid (0.22 g, 55%): mp 186–189°C; $[\alpha]_D$ −10.0° (*c* 1.0, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.76 (t, 3 H, *J* 7.5 Hz, C*H*$_3$CH$_2$), 1.49 (m, 2 H, CH$_3$C*H*$_2$), 2.03 (t, 2 H, *J* 7.5 Hz, C*H*$_2$CO), 2.04, 2.05, and 2.06 (3 s, 9 H, 3 OAc), 5.48 (d, 1 H, *J* 10.0 Hz, H-1), 7.13–7.74 (7 H, Ar); ^{13}C NMR (126 MHz, $CDCl_3$): δ 13.5 (*C*H$_3$CH$_2$), 19.1 (CH$_3$*C*H$_2$), 20.7 and 20.8 (2 C) (3 OCO*C*H$_3$), 38.7 (*C*H$_2$CO), 98.9 (C-1), 111.3, 118.9, 124.6, 126.6, 127.1, 127.8, 129.6, 130.1, 134.3, and 155.0 (Ar), 169.6, 170.7, 170.9, and 173.7 (3 O*C*OCH$_3$, N*C*OCH$_3$); HRMS (ES): Calcd for $C_{26}H_{32}NO_9$ ([M + H]$^+$): 502.2077. Found: 502.2058.

2-Naphthyl 2-butanamido-2-deoxy-β-D-glucopyranoside (**11**)

A sample (0.25 g, 0.50 mmol) of 2-naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-β-D-glucopyranoside prepared above was de-*O*-acetylated following Method A to give **11** (0.17 g, 92%) as a white solid: ^{1}H NMR (500 MHz, CD_3OD): δ 0.95 (t, 3 H, *J* 7.5 Hz, C*H*$_3$CH$_2$), 1.68 (m, 2 H, CH$_3$C*H*$_2$), 2.24 (m, 2 H, C*H*$_2$CO), 5.24 (d, 1 H, H-1), 7.20–7.80 (7 H, Ar); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.4 (*C*H$_3$CH$_2$), 18.7 (CH$_3$*C*H$_2$), 37.8 (*C*H$_2$CO), 99.3 (C-1), 110.5, 118.6, 124.1, 126.4, 126.9, 127.5, 129.1, 129.3, 134.0, and 155.3 (Ar), 172.1 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{20}H_{25}NO_6Na$ ([M + Na]$^+$): 398.1580. Found: 398.1573.

3-Isoquinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside

The title compound was prepared from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) (0.45 g, 1.22 mmol) and 3-hydroxyisoquinoline following the representative procedure for the synthesis of aryl glycosides and was obtained as a pale yellow solid (0.22

g, 38%): mp 152–155°C; $[\alpha]_D$ +15.0° (*c* 1.1, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 1.88 (s, 3 H, NAc), 2.05, 2.08, and 2.09 (3 s, 9 H, 3 OAc), 6.12 (d, 1 H, *J* 10.0 Hz, H-1), 7.19–8.87 (6 H, Ar); ^{13}C NMR (126 MHz, $CDCl_3$): δ 20.7, 20.8, and 20.9 (3 OCO*C*H_3), 23.4 (NCO*C*H_3), 95.4 (C-1), 104.7, 125.5, 126.1, 126.4, 127.6, 130.9, 139.3, 150.3, and 158.5 (Ar), 169.5, 170.4, 170.8, and 171.2 (3 O*C*OCH$_3$, N*C*OCH$_3$); HRMS (ES): Calcd for $C_{23}H_{27}N_2O_9$ ([M + H]$^+$): 475.1717. Found: 475.1726.

3-Isoquinolinyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**24**)

A sample (73 mg, 0.15 mmol) of 3-isoquinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside prepared above was de-*O*-acetylated following Method B to give **24** (42 mg, 78%) as a pale yellow solid: mp 216–218°C; ^1H NMR (400 MHz, DMSO-d_6): δ 1.75 (s, 3 H, NAc), 5.86 (d, 1 H, *J* 8.5 Hz, H-1), 7.20–9.07 (6 H, Ar); ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.1 (NCO*C*H_3), 95.8 (C-1), 102.9, 125.1, 125.5, 125.7, 127.6, 130.8, 138.7, 150.7, and 159.1 (Ar), 169.2 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{17}H_{21}N_2O_6$ ([M + H]$^+$): 349.1399. Found: 349.1403.

6-Quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside

The title compound was prepared from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) (0.46 g, 1.27 mmol) and 6-hydroxyquinoline following the representative procedure for the synthesis of aryl glycosides and was obtained as a white solid (0.25 g, 41%): mp 195–196°C; $[\alpha]_D$ −10.9° (*c* 0.6, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 1.99 (s, 3 H, NAc), 2.06, 2.08, and 2.10 (3 s, 9 H, 3 OAc), 5.45 (d, 1 H, *J* 8.2 Hz, H-1), 7.37–8.83 (6 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.6, 20.7, and 20.7 (3 OCO*C*H_3), 23.4 (NCO*C*H_3), 98.7 (C-1), 110.9, 121.6, 122.3, 129.0, 131.0, 135.2, 145.1, 149.0, and 154.8 (Ar), 169.4, 170.6, 170.6, and 170.8 (3 O*C*OCH$_3$, N*C*OCH$_3$); HRMS (ES): Calcd for $C_{23}H_{27}N_2O_9$ ([M + H]$^+$): 475.1717. Found: 475.1731.

6-Quinolinyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**25**)

A sample (28 mg, 0.15 mmol) of 6-quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside prepared above was de-*O*-acetylated following Method B to give **25** (18 mg, 91%) as a white solid: ^1H NMR (500 MHz, DMSO-d_6/$CDCl_3$): δ 1.83 (s, 3 H, NAc), 5.17 (d, 1 H, *J* 8.5 Hz, H-1), 7.41–8.78 (6 H, Ar); ^{13}C NMR (126 MHz, DMSO-d_6/$CDCl_3$): δ 23.1 (NCO*C*H_3), 99.2 (C-1), 110.4, 121.7, 122.0, 128.7, 130.4, 135.1, 144.1, 148.6, and 155.1 (Ar), 169.3 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{17}H_{21}N_2O_6$ ([M + H]$^+$): 349.1399. Found: 349.1400.

8-Quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside

The title compound was prepared from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) (0.45 g, 1.34 mmol) and 8-hydroxyquinoline following the representative procedure for the synthesis of aryl glycosides and was obtained as a white solid (0.27 g, 42%): mp 173–175°C; $[\alpha]_D$ −27.2° (*c* 2.1, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 2.03, 2.04, 2.09, and 2.11 (4 s, 12 H, 3 OAc, NAc), 5.43 (d, 1 H, H-1), 7.51–8.92 (6 H, Ar); ^{13}C NMR (126 MHz, $CDCl_3$): δ 20.8, 20.9, and 21.0 (3 OCO*C*H_3), 23.6 (NCO*C*H_3), 101.9 (C-1), 121.6, 121.7, 124.8, 127.0, 130.3, 137.3, 141.1, 149.5, and 152.1 (Ar), 169.5, 170.8, 171.1, and 171.5 (3 O*C*OCH$_3$, N*C*OCH$_3$); HRMS (ES): Calcd for $C_{23}H_{27}N_2O_9$ ([M + H]$^+$): 475.1717. Found: 475.1719.

8-Quinolinyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**26**)

A sample (82 mg, 0.17 mmol) of 8-quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside prepared above was de-*O*-acetylated following Method B to give **26** (49 mg, 81%) as a white solid: mp 247–248°C (dec); ^1H NMR (400 MHz, DMSO-d_6): δ 1.80 (s, 3 H, NAc), 5.39 (d, 1 H, *J* 8.1 Hz, H-1), 7.39–8.88 (6 H, Ar); ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.1 (NCOC*H*$_3$), 99.1 (C-1), 113.8, 121.5, 121.7, 126.6, 129.0, 135.8, 139.9, 149.3, and 153.1 (Ar), 169.6 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{17}H_{21}N_2O_6$ ([M + H]$^+$): 349.1399. Found: 349.1398.

5-Chloro-8-quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside

The title compound was prepared from 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) (1.38 g, 3.77 mmol) and 5-chloro-8-hydroxyquinoline following the representative procedure for the synthesis of aryl glycosides and was obtained as a white solid (1.32 g, 68%): mp 180–182°C; $[\alpha]_D$ −11.5° (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ 2.04, 2.04, 2.09, and 2.11 (4 s, 12 H, 3 OAc, NAc), 5.40 (d, 1 H, *J* 10.0 Hz, H-1), 7.48–8.97 (5 H, Ar); ^{13}C NMR (126 MHz, $CDCl_3$): δ 20.7, 20.9, and 21.0 (3 OCO*C*H_3), 23.6 (NCO*C*H_3), 101.7 (C-1), 121.2, 122.5, 126.9, 127.5, 127.9, 134.3, 141.8, 150.1, and 151.2 (Ar), 169.5, 170.8, 171.0, and 171.3 (3 O*C*OCH$_3$,

N*C*OCH$_3$); HRMS (ES): Calcd for $C_{23}H_{26}ClN_2O_9$ ([M + H]$^+$): 509.1326. Found: 509.1329.

5-Chloro-8-quinolinyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**27**)

A sample (183 mg, 0.36 mmol) of 5-chloro-8-quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside prepared above was de-*O*-acetylated following Method B to give **27** (117 mg, 85%) as a white solid: mp 244–246°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.78 (s, 3H, NCOC*H*$_3$), 5.40 (d, 1 H, *J* 8.2 Hz, H-1), 7.40–8.98 (5H, Ar); ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.1 (NCOC*H*$_3$), 99.1 (C-1), 113.7, 122.5, 123.0, 126.2, 126.7, 132.2, 140.4, 150.2, and 152.5 (Ar), 169.6 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{17}H_{20}ClN_2O_6$ ([M + H]$^+$): 383.1009. Found: 383.1002.

2-Quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside

To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**51**) (0.75 g, 2.04 mmol), 2-quinolinethiol (0.66 g, 4.08 mmol), and tetra-*n*-butylammonium hydrogensulfate (0.69 g, 2.04 mmol) in ethyl acetate (7.5 mL) was added an aqueous 1.5 M solution of sodium carbonate (7.5 mL). The resulting two-phase system was then stirred for 2.5 h at rt. The mixture was diluted with ethyl acetate (75 mL) and the organic phase washed sequentially with a saturated aqueous solution of sodium hydrogencarbonate (2 × 50 mL), water (2 × 50 mL), and brine (50 mL), and dried (Na_2SO_4). The organic extract was filtered and the filtrate was concentrated to yield a crude solid which was recrystallized (ethyl acetate–hexanes) to give the title compound (0.69 g, 70%) as a white solid: mp 186–187°C; $[\alpha]_D$ −8.5° (*c* 0.9, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.83, 1.98, 2.08, 2.08 (4s, 12H, 3 OAc, NAc), 6.01 (d, 1 H, *J* 10.0 Hz, H-1), 7.27–7.99 (6H, Ar); ^{13}C NMR (126 MHz, $CDCl_3$): δ 20.8, 20.8, and 20.9 (3 OCO*C*H$_3$), 23.3 (NCO*C*H$_3$), 82.3 (C-1), 121.4, 126.1, 126.5, 127.9, 128.0, 130.3, 136.4, 148.1, and 156.9 (Ar), 169.4, 170.4, 170.8, and 171.4 (3 O*C*OCH$_3$, N*C*OCH$_3$); HRMS (ES): Calcd for $C_{23}H_{27}N_2O_8S$ ([M + H]$^+$): 491.1488. Found: 491.1464.

2-Quinolinyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside (**28**)

A sample (135 mg, 0.28 mmol) of 2-quinolinyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside prepared above was de-*O*-acetylated following Method B to give **28** (86 mg, 86%) as a white solid: mp 208–210°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.80 (s, 3H, NAc), 5.54 (d, 1 H, *J* 9.4 Hz, H-1), 7.42–8.21 (6H, Ar); ^{13}C NMR (126 MHz, DMSO-d_6): δ 23.0 (NCO*C*H$_3$), 82.7 (C-1), 120.5, 125.6, 125.8, 127.6, 128.0, 130.1, 136.5, 147.4, and 158.5 (Ar), 169.2 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{17}H_{20}N_2O_5SNa$ ([M + Na]$^+$): 387.0990. Found: 387.0990.

Benzyl 2-acetamido-2-deoxy-6-thio-β-D-glucopyranoside (**29**)

To a solution of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) (164 mg, 0.53 mmol) in pyridine (4 mL) at 0°C was added *p*-toluenesulfonyl chloride (124 mg, 0.63 mmol) and the solution stirred for 16 h at 0°. Ethyl acetate (1 mL) was added followed by a saturated aqueous solution of sodium hydrogencarbonate (1 mL). The reaction mixture was stirred for an additional 5 min and then diluted with ethyl acetate (20 mL). The organic phase was extracted with a saturated solution of sodium hydrogencarbonate (20 mL), and the aqueous layer was extracted with chloroform (20 mL). The organic extract was dried (Na_2SO_4) and concentrated to give benzyl 2-acetamido-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (**53**) as a crude solid (213 mg, 87%) that was used without further purification.

To a solution of benzyl 2-acetamido-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (**53**) (213 mg, 0.40 mmol) in pyridine (2 mL) was added acetic anhydride (2 mL) and the solution stirred for 16 h at rt. The solvent was removed under reduced pressure and the resulting residue was purified by column chromatography on silica gel {4:1 (v/v) ethyl acetate–hexanes} to give benzyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside as a white solid (215 mg, 86%): $[\alpha]_D$ +25.8° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): 1.92 (s, 3 H, NAc), 2.02 (2s, 6 H, 2 OAc), 2.46 (s, 3 H, C*H*$_3$Ph), 4.53 (d, 1 H, *J* 12.0 Hz, one OC*H*$_2$Ph), 4.58 (d, 1 H, *J* 8.4 Hz, H-1), 4.83 (d, 1 H, *J* 12.0 Hz, one OC*H*$_2$ Ph), 7.28–7.83 (9 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.6, 20.7 (2 OCO*C*H$_3$), 21.7 (NCO*C*H$_3$), 23.3 (*C*H$_3$Ph), 99.2 (C-1), 170.0, 170.1, and 170.9 (2 O*C*OCH$_3$, N*C*OCH$_3$); HRMS (ES): Calcd for $C_{26}H_{32}NO_{10}S$ ([M + H]$^+$): 550.1746. Found: 550.1764.

To a solution of benzyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (143 mg, 0.26 mmol) in DMF (5 mL) was added potassium thioacetate (148 mg, 1.30 mmol) and the reaction mixture stirred for 16 h at 80°C. The solution was cooled to rt and diluted with ethyl acetate (20 mL). The organic layer was washed sequentially with a saturated aqueous solution of sodium hydrogencarbonate (2 × 20 mL), water (20 mL), brine (20 mL), dried (Na_2SO_4), and concentrated. The resulting residue was purified by column chromatography on silica gel (ethyl acetate) to give benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-6-thio-β-D-glucopyranoside as a white solid (101 mg, 86%): $[\alpha]_D$ +28.1° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.92

(s, 3, NAc), 2.02, 2.08 (2 s, 6 H, 2 O Ac), 2.38 (s, 3 H, SAc), 4.57 (d, 1 H, *J* 8.4 Hz, H-1), 4.61 (d, 1 H, *J* 12.0 Hz, one OC*H*$_2$Ph), 4.88 (d, 1 H, *J* 12.4 Hz, one OC*H*$_2$Ph), 7.28–7.35 (5 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.6 and 20.7 (2 O*C*OCH$_3$), 23.2 (N*C*OCH$_3$), 30.4 and 30.5 (SCO*C*H$_3$, C-6), 99.1 (C-1), 128.0 (2 C), 128.4, and 136.8 (Ar), 169.6, 170.0, and 170.9 (2 O*C*OCH$_3$, N*C*OCH$_3$), 194.8 (S*C*OCH$_3$); HRMS (ES): Calcd for $C_{21}H_{28}NO_8S$ ([M + H]$^+$): 454.1535. Found: 454.1547.

To a solution of benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-6-thio-β-D-glucopyranoside (50 mg, 0.11 mmol) in methanol (1.5 mL) was added a solution of potassium hydroxide (41 mg, 0.73 mmol) in methanol (1.5 mL) and the resulting mixture stirred for 1 h at rt. The mixture was neutralized using IR-120 (hydrogen form) ion exchange resin (100 mg) and stirred for an additional 15 min. The solution was then filtered and the filtrate concentrated to give benzyl 2-acetamido-2-deoxy-6-thio-β-D-glucopyranoside (**29**) as white solid (24 mg, 67%): ^{1}H NMR (400 MHz, CD_3OD): δ 1.99 (s, 3 H, NAc), 4.42 (d, 1 H, *J* 8.4 Hz, H-1), 4.62 (d, 1 H, *J* 12.4 Hz, one OC*H*$_2$Ph), 4.88 (d, 1 H, *J* 12.2 Hz, one OC*H*$_2$Ph), 7.25–7.38 (5H, Ar); ^{13}C NMR (100 MHz, CD_3OD): δ 23.1 (NCO*C*H$_3$), 101.8 (C-1), 173.9 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{15}H_{21}NO_5SNa$ ([M + Na]$^+$): 350.1038. Found: 350.1051.

2-Naphthyl 2-butanamido-2-deoxy-1,6-dithio-β-D-glucopyranoside (**12**)

To a solution of 2-naphthyl 2-butanamido-2-deoxy-1-thio-β-D-glucopyranoside (**10**) (168 mg, 0.43 mmol) in pyridine (4 mL) at 0°C was added *p*-toluenesulfonyl chloride (92 mg, 0.47 mmol) and the solution stirred for 4 h at 0°C. Ethyl acetate (1 mL) was added followed by a saturated aqueous solution of sodium hydrogencarbonate (1 mL). The reaction mixture was stirred for an additional 5 min and then diluted with ethyl acetate (10 mL). The organic phase was extracted with a saturated aqueous solution of sodium hydrogencarbonate (10 mL), and the aqueous layer was then extracted with chloroform (10 mL). The organic extract was dried (Na_2SO_4) and concentrated to give 2-naphthyl 2-butanamido-2-deoxy-1-thio-6-O-*p*-toluenesulfonyl-β-D-glucopyranoside as a crude solid (197 mg, 84%) that was used without further purification.

To a solution of 2-naphthyl 2-butanamido-2-deoxy-1-thio-6-O-*p*-toluenesulfonyl-β-D-glucopyranoside (197 mg, 0.36 mmol) in pyridine (3 mL) was added acetic anhydride (2 mL) and the solution stirred for 16 h at rt. The solvent was removed under reduced pressure, and the resulting residue was purified by column chromatography on silica gel {2:1 (v/v) ethyl acetate–hexanes} to give 2-naphthyl 3,4-di-*O*-acetyl-2-butanamido-2-deoxy-1-thio-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside as a white solid (192 mg, 85%): $[\alpha]_D$ −4.4° (*c* 1.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.97 (t, 3 H, *J* 7.0 Hz, C*H*$_3$CH$_2$), 1.67 (m, 2 H, CH$_3$C*H*$_2$), 2.00, 2.02, and 2.04 (3 s, 9 H, 2 OAc, NAc), 2.17 (m, 2H, C*H*$_2$CO), 2.41 (s, 3, C*H*$_3$Ph), 4.94 (d, 1 H, *J* 10.0 Hz, H-1), 7.28–7.80 (11 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.7 (*C*H$_3$CH$_2$), 19.0 (CH$_3$*C*H$_2$), 20.6 and 20.7 (2 OCO*C*H$_3$), 21.6 (*C*H$_3$Ph), 38.7 (*C*H$_2$CO), 86.9 (C-1), 126.5, 126.7, 127.7, 127.8, 128.1 (2 C), 128.5, 129.1, 129.9 (2 C), 130.8, 131.7, 132.3, 132.6, 133.6, 145.1 (Ar), 169.5 and 170.9 (2 O*C*OCH$_3$), 173.0 (N*C*OCH$_3$); HRMS (ES): Calcd for $C_{31}H_{36}NO_9S_2$ ([M + H]$^+$): 630.1832. Found: 630.1844.

To a solution of 2-naphthyl 3,4-di-*O*-acetyl-2-butanamido-2-deoxy-1-thio-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (168 mg, 0.27 mmol) in DMF (6 mL) was added potassium thioacetate (152 mg, 1.33 mmol) and the mixture stirred for 16 h at 80°C. The solution was cooled to rt and diluted with ethyl acetate (50 mL). The organic layer was washed sequentially with a saturated aqueous solution of sodium hydrogencarbonate (2 × 50 mL), water (50 mL), and brine (50 mL), dried (Na_2SO_4), and concentrated. The resulting residue was purified by column chromatography on silica gel {2:1 (v/v) ethyl acetate–hexanes} to give 2-naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-1,6-dithio-β-D-glucopyranoside as a white solid (105 mg, 74%): $[\alpha]_D$ +20.1° (*c* 1.9, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.98 (t, 3 H, *J* 7.4 Hz, C*H*$_3$CH$_2$), 1.67 (m, 2 H, CH$_3$C*H*$_2$), 2.03 and 2.05 (2s, 6 H, 2 OAc), 2.20 (m, 2 H, C*H*$_2$CO), 2.30 (s, 3 H, SAc), 4.90 (d, 1 H, *J* 10.4 Hz, H-1), 7.49–8.04 (7 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.7 (*C*H$_3$CH$_2$), 19.0 (CH$_3$*C*H$_2$), 20.4 and 20.7 (2 OCO*C*H$_3$), 30.4 and 30.5 (SCO*C*H$_3$, C-6), 38.7 (*C*H$_2$CO), 86.9 (C-1), 126.5, 126.6, 127.6, 127.7, 128.4, 129.7, 130.1, 131.6, 132.7, and 133.6 (Ar), 169.7, 171.0, and 173.0 (2 O*C*OCH$_3$, N*C*OCH$_3$), 194.7 (S*C*OCH$_3$); HRMS (ES): Calcd for $C_{26}H_{31}NO_7S_2Na$ ([M + Na]$^+$): 556.1440. Found: 556.1431.

To a solution of 2-naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-1,6-dithio-β-D-glucopyranoside (52 mg, 0.10 mmol) in methanol (1 mL) was added a solution of potassium hydroxide (35 mg, 0.64 mmol) in methanol (2 mL) and the resulting mixture stirred for 1 h at rt. The mixture was then neutralized using IR-120 (hydrogen form) ion exchange resin (100 mg) and stirred for an additional 15 min. The suspension was filtered and the filtrate concentrated to give 2-naphthyl 2-butanamido-2-deoxy-1,6-dithio-β-D-glucopyranoside (**12**) as a white solid (27 mg, 68%):$[\alpha]_D$ +78° (*c* 2.0, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.92 (t, 3 H, *J* 7.4 Hz, C*H*$_3$CH$_2$), 1.57 (m, 2 H, CH$_3$C*H*$_2$), 2.08 (t, 2 H, *J* 7.0 Hz, C*H*$_2$CO), 4.90 (d, 1 H, *J* 10.4 Hz, H-1), 7.25–8.00 (7H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1 (*C*H$_3$CH$_2$), 19.2 (CH$_3$*C*H$_2$), 38.3 (*C*H$_2$CO), 54.7 (C-2), 87.2 (C-1); HRMS (ES): Calcd for $C_{20}H_{25}NO_4S_2Na$ ([M + Na]$^+$): 430.1123. Found: 430.1118.

2-Naphthyl 2-butanamido-2-deoxy-6-thio-β-D-glucopyranoside (**15**)

To a solution of 2-naphthyl 2-butanamido-2-deoxy-β-D-glucopyranoside (**11**) (122 mg, 0.32 mmol) in pyridine (3 mL) at 0°C was added *p*-toluenesulfonyl chloride (76 mg, 0.39 mmol) and the solution stirred for 3 h at 0°C. Ethyl acetate (1 mL) was added followed by a saturated aqueous solution of sodium hydrogencarbonate (1 mL). The reaction mixture was stirred for an additional 5 min and then diluted with ethyl acetate (15 mL). The organic phase was extracted with a saturated solution of sodium hydrogencarbonate (15 mL), and the aqueous layer was then extracted with chloroform (15 mL). The organic extract was dried (Na_2SO_4) and concentrated to give 2-naphthyl 2-butanamido-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside as a crude solid (138 mg, 77%) that was used without further purification.

To a solution of 2-naphthyl 2-butanamido-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (138 mg, 0.26 mmol) in pyridine (3 mL) was added acetic anhydride (3 mL) and the solution stirred for 16 h at rt. The solvent was removed under reduced pressure, and the resulting residue was purified by column chromatography on silica gel (ethyl acetate) to give 2-naphthyl 3,4-di-*O*-acetyl-2-butanamido-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside as a white solid (192 mg, 85%): $[\alpha]_D$ –10.8° (*c* 2.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.81 (t, 3 H, *J* 7.4 Hz, C*H*$_3$CH$_2$), 1.55 (m, 2H, CH$_3$C*H*$_2$), 2.00 and 2.05 (2s, 6 H, 2 OAc), 2.08 (m, 2 H, C*H*$_2$CO), 2.28 (s, 3 H, C*H*$_3$Ph), 5.40 (d, 1 H, *J* 8.4 Hz, H-1), 7.06–7.79 (11 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.5 (*C*H$_3$CH$_2$), 19.1 (CH$_3$*C*H$_2$), 20.6 and 20.7 (2 OCO*C*H$_3$), 21.5 (*C*H$_3$Ph), 38.6 (*C*H$_2$CO), 98.7 (C-1), 111.2, 118.7, 124.6, 126.6, 127.3, 127.6, 128.0 (2C), 129.4, 129.8, 130.0 (2 C), 132.1, 134.2, 145.1, and 154.7 (Ar), 169.6 and 170.8 (2 O*C*OCH$_3$), 173.5 (N*C*OCH$_2$); HRMS (ES): Calcd for $C_{31}H_{35}NO_{10}SNa$ ([M + Na]$^+$): 636.1879. Found: 636.1878.

To a solution of 2-naphthyl 3,4-di-*O*-acetyl-2-butanamido-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (119 mg, 0.19 mmol) in DMF (4 mL) was added potassium thioacetate (111 mg, 0.97 mmol) and the reaction mixture stirred for 16 h at 80°C. The solution was then cooled to rt and diluted with ethyl acetate (20 mL). The organic layer was washed sequentially with a saturated aqueous solution of sodium hydrogencarbonate (2 × 20 mL), water (20 mL), and brine (20 mL), dried (Na_2SO_4), and concentrated. The resulting residue was purified by column chromatography on silica gel {2:1 (v/v) ethyl acetate–hexanes} to give 2-naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-6-thio-β-D-glucopyranoside as a white solid (79 mg, 79%): $[\alpha]_D$ +36.4° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (t, 3 H, *J* 7.4 Hz, C*H*$_3$CH$_2$), 1.59 (m, 2 H, CH$_3$C*H*$_2$), 2.08 (s, 3 H, OAc), 2.11 (m, 2 H, C*H*$_2$CO), 2.14 (s, 3 H, OAc), 2.38 (s, 3 H, SAc), 5.34 (d, 1 H, *J* 8.4 Hz, H-1), 7.16–7.81 (7 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.5 (*C*H$_3$CH$_2$), 19.1 (CH$_3$*C*H$_2$), 20.7 and 20.8 (2 OCO*C*H$_3$), 30.4 and 30.5 (C-6, SCO*C*H$_3$), 38.7 (*C*H$_2$CO), 99.1 (C-1), 111.4, 118.9, 124.5, 126.5, 127.1, 127.7, 129.5, 130.0, 134.2, and 154.9 (Ar), 169.8 and 170.8 (2 O*C*OCH$_3$), 173.3 (N*C*OCH$_2$), 194.7 (S*C*OCH$_3$); HRMS (ES): Calcd for $C_{26}H_{31}NO_8SNa$ ([M + Na]$^+$): 540.1668. Found: 540.1650.

To a solution of 2-naphthyl 3,4,6-tri-*O*-acetyl-2-butanamido-2-deoxy-6-thio-β-D-glucopyranoside (45 mg, 0.09 mmol) in methanol (1.5 mL) was added a solution of potassium hydroxide (32 mg, 0.57 mmol) in methanol (1.5 mL) and the resulting mixture stirred for 1 h at rt. The reaction was neutralized using IR-120 (hydrogen form) ion exchange resin (100 mg) and stirred for an additional 15 in. The suspension was diluted with methanol (20 mL), filtered, and the filtrate concentrated to give 2-naphthyl 2-butanamido-2-deoxy-6-thio-β-D-glucopyranoside (**15**) as a white solid (32 mg, 94%): ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (t, 3 H, *J* 7.3 Hz, C*H*$_3$CH$_2$), 1.53 (m, 2 H, CH$_3$C*H*$_2$), 2.06 (m, 2 H, C*H*$_2$CO), 5.11 (d, 1 H, *J* 8.5 Hz, H-1), 7.13–7.85 (7 H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.5 (*C*H$_3$CH$_2$), 18.7 (CH$_3$*C*H$_2$), 37.8 (*C*H$_2$CO), 99.6 (C-1), 110.5, 118.5, 124.1, 126.4, 126.7, 127.4, 129.1, 129.2, 133.9, and 155.2 (Ar), 172.1 (N*C*OCH$_2$); HRMS (ES): Calcd for $C_{20}H_{25}NO_5SNa$ ([M + Na]$^+$): 414.1351. Found: 414.1345.

Benzyl 2-butanamido-2-deoxy-β-D-glucopyranoside (**43**)

The general method of Vauzeilles et al. [30] was employed for the synthesis of **43**. To a solution of 2-butanamido-2-deoxy-D-glucopyranose [42] (3.15 g, 12.6 mmol) in dry DMF (40 mL) was added to a 60% suspension of sodium hydride in mineral oil (0.65 g, 16.4 mmol) and the suspension stirred under nitrogen until the evolution of H_2 gas had ceased (1 h). To the mixture was added benzyl bromide (6.48 g, 37.8 mmol) and the mixture stirred for 4 h. Water (100 mL) was added and the mixture extracted with ethyl acetate (2 × 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated. The resulting residue was frationated by column chromatography on silica gel {40:1 (v/v) ethyl acetate–methanol} to give **43** as a white solid (2.34 g, 55%): $[\alpha]_D$ –95.0° (*c* 1.0, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 0.91 (t, 3 H, J 7.5 Hz, C*H*$_3$CH$_2$), 1.59 (m, 2 H, CH$_3$C*H*$_2$), 2.15 (m, 2 H, C*H*$_2$CO), 4.46 (d, 1 H, *J* 9.25Hz, H-1), 7.22–7.55 (5 H, Ar); ^{13}C NMR (126 MHz, CD_3OD): δ 14.1 (*C*H$_3$CH$_2$), 20.4 (CH$_3$*C*H$_2$), 39.4 (*C*H$_2$CO), 101.9 (C-1), 128.6, 128.9 (2 C), 129.3 (2 C), and 139.1 (Ar), 176.6 (N*C*OCH$_2$); HRMS (ES): Calcd for $C_{17}H_{25}NO_6Na$ ([M + Na]$^+$): 362.1580. Found: 362.1594.

Gal-transferase assays

The standard assay mixtures for β4GalT1 activity contained in a total volume of 40 μL: acceptor substrate as indicated in the Tables, 0.15 to 0.68 μg β4GalT1 (1 to 4 mU), 0.125 M MES buffer (pH 7), 12.5 mM $MnCl_2$, 50 μg bovine serum albumin and 1 mM UDP-[^{3}H]Gal (1,000–4,000 cpm/nmol). Control assays lacked acceptor substrate. The standard β4GalT assays contained GlcNAcβ-Bn (**1**), or GlcNAc (**2**) as the control acceptor substrate. When using compounds soluble in methanol–water or isopropanol–water mixtures, the same amount of solvent was added to all assays of the series. No significant inhibition of activity was noted up to 20% methanol, or up to 7% isopropanol in the assay. Mixtures were incubated for 30 min at 37°C. Reactions were stopped by the addition of 600 μL cold water and mixtures were passed through 0.4 mL columns of AG1 $\times$ 8 (Cl^--form). Columns were then washed twice with 600 μL water. 5 mL of scintillation fluid (Ready Safe) was added to the combined eluates and radioactivity was determined by scintillation counting. For HPLC analysis, eluates from AG1 $\times$ 8 (Bio-Rad) were dried, residues taken up in water or methanol (depending on the solubility of the substrates), and aliquots were injected into an HPLC system. HPLC separations of substrates and enzyme products were carried out using C18 or amine columns using acetonitrile–water mixtures as the mobile phase at a flow rate of 1 mL/min [46]. Generally, compounds with hydrophobic groups were separated on C18 columns, and hydrophilic compounds such as mono- and oligo-saccharides were separated using an amine column.

Kinetic parameters

Compounds that appeared to be good substrates were analyzed kinetically, and the data processed using non-linear regression programs ENZFIT, Leonora and EZ-FIT [47], as well as by manual graphical methods. Inhibitors were tested kinetically, and parameters calculated by reciprocal plots using EZ-FIT and Leonora programs [48].

Results

Role of the glycosidic linkage

The standard assays for β4GalT utilized GlcNAcβ-Bn (**1**) as the acceptor because of the high efficacy of this substrate. Under standard assay conditions, galactosyl transfer to **1** was linear with time for at least 30 min and was proportional to enzyme concentration up to 0.7 μg enzyme protein. The kinetics of transfer to **1** were complex and this acceptor demonstrated strong substrate inhibition with a K_{si} of 3.58 mM (Table 1). The kinetics were analyzed using non-linear regression methods with the best fit used to differentiate between Michaelis–Menton kinetics and substrate inhibition. The K_m for **1** was 1.38 mM with a V_{max} of 123 μmol/h/mg. The rates of galactosyl transfer to the other acceptors were then compared with, or normalized to, the rate with **1** within each set of experiments. The preference of β4GalT for the anomeric configuration of GlcNAc in the acceptor substrate was established in assays using the α- or the β-configuration of both of the glycosides GlcNAc-Bn and GlcNAc-(*p*-NO_2Ph) (Figure 1, Table 2). The rates of transfer to the α-anomers of the GlcNAc derivatives were found to be only 6–9% of the activity when compared with the respective β-anomers at 2 mM substrate concentration (Table 2). The K_m for GlcNAcβ-Bn (**1**) was 5-fold lower than the K_m for the α-anomer **3** with a 7.4-fold higher V_{max} (Table 1), suggesting a better fit for the β-anomer in the substrate binding site. Interestingly, the kinetics for the α-anomer gave a best fit to Michaelis–Menten kinetics (Figure 1). However, it was not practical to assay the enzyme at higher concentrations of the α-anomer where substrate inhibition may have been clearly defined. Although there was a preference for the β-configuration over the α-configuration in the case of the *O*-glycosyl compounds, apparently the *O*-glycosidic linkage was not required for acceptor binding to the active site. Thus, a *C*-glycosyl derivative (**6**) of GlcNAcβ and the *S*-glycosyl compound 1-thioGlcNAcβ-Et (**7**) were slightly more active (1.26-fold and 1.27-fold, respectively) than **1** when tested at 2 mM concentration (Table 2). This aspect was further confirmed by the observation that the phenyl *O*- and *S*-β-glycosyl compounds, GlcNAcβ-Ph (**13**) and 1-thioGlcNAcβ-Ph (**14**), were equivalent substrates with only a 15% difference in rate at 2 mM. Thus, apparently the *O*-glycosidic linkage is not a required structural feature for a fully active substrate, and GlcNAc residues linked by sulfur or carbon are also well recognized by the enzyme.

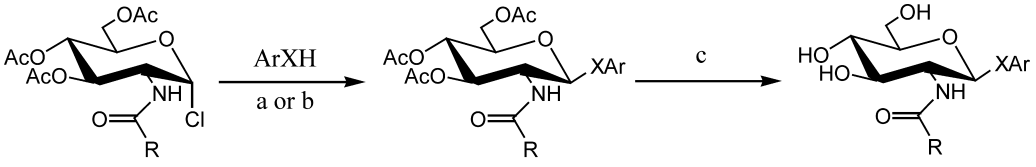
Role of the aglycone group

A series of substrates containing aglycones of varying hydrophobicity and size were tested as substrates. Although free GlcNAc is an acceptor for galactose, it was found to have a V_{max} of only 45% of that of the benzyl glycoside **1** with a K_m 2.5-fold higher (Table 1, Figure 1), demonstrating that a hydrophobic aglycone in the substrate promotes binding to the active site of the enzyme. However, the presence of the benzyl aglycone also had the secondary effect of allowing the substrate to bind to the enzyme in a non-productive complex, resulting in significant substrate inhibition (Figure 1, Table 1). Other acceptors having aromatic aglycone groups, namely *N*-butyrylGlcNβ-Bn (**43**) and 1-thioGlcNAcβ-Ph (**14**), were also good substrates showing significant substrate inhibition with K_{si} values of 8.13 and 0.34 mM, respectively (Table 1). For comparison purposes, the K_m and V_{max} values for other

Table 1 Kinetic parameters for glucosamine analogs as β4Gal-transferase acceptors

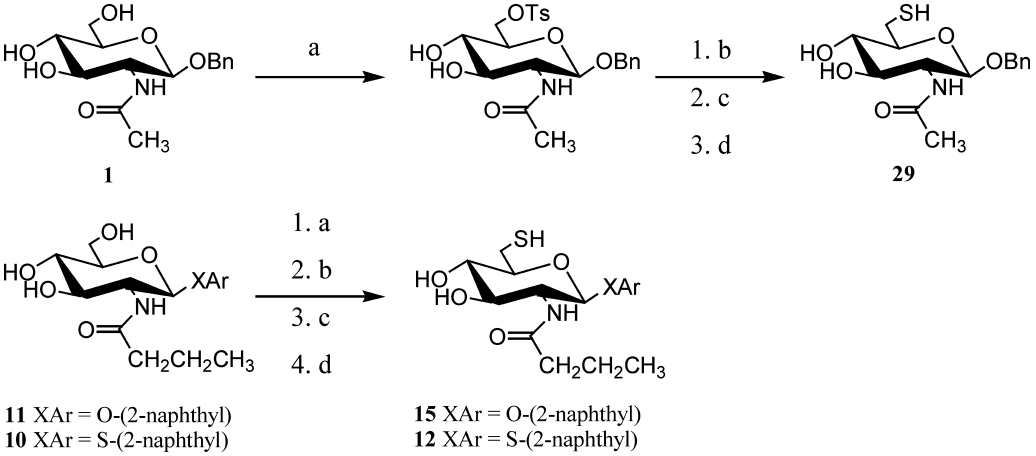
Compound		K_m	V_{max}	Kinetic efficiency	K_{si}
No.	Structure	(mM)	(μmol/h/mg)	V_{max}/K_m	(mM)
Substrate inhibition:					
1	GlcNAcβ-Bn	1.38	123	89	3.58
14	1-ThioGlcNAcβ-Ph	0.64	113	177	0.34
43	*N*-ButyrylGlcNβ-Bn	1.00	138	138	8.13
Michaelis–Menten kinetics:					
2	GlcNAc	3.51	55.6	16	
3	GlcNAcα-Bn	6.65	16.6	2	
4	GlcNAcβ-(*p*-NO_2Ph)	0.15	13.7	91	
13	GlcNAcβ-Ph	0.82	47	57	
19	GlcNAcβ-(11-phenoxyundecyl)	0.34	4.3	13	
24	GlcNAcβ-(3-isoquinolinyl)	0.11	12.6	115	
25	GlcNAcβ-(6-quinolinyl)	0.23	19.8	86	
26	GlcNAcβ-(8-quinolinyl)	0.25	6.5	26	
27	GlcNAcβ-[8-(5-chloroquinolinyl)]	0.44	6.6	15	
28	1-ThioGlcNAcβ-(2-quinolinyl)	0.09	7.3	81	
30	3-Deoxy-3-fluoroGlcNAc	10.6	100	9	
44	*N*-*p*-ToluenesulfonylGlcN	25.2	22.3	1	
45	*N*-MethanesulfonylGlcN	25.2	22.3	1	

Compounds above were used as substrates in β4GalT assays, as described in the Methods section, with 1.2 mM UDP-Gal concentration and at least five concentrations of acceptor substrate. Kinetic constants and mechanisms were determined by non-linear regression with EZFIT, Leonora and ENZFIT, as well as by graphical methods of initial rates.



R = CH_3 or $CH_2CH_2CH_3$
Ar = naphthyl, quinolinyl or isoquinolinyl
X = O or S

Scheme 1 Synthesis of naphthyl, quinolinyl and isoquinolinyl glycosides and thioglycosides. Reagents and conditions: (a) tetra-*n*-butylammonium bromide, CH_2Cl_2/1.0 M NaOH; (b) tetra-*n*-butylammonium hydrogensulfate, EtOAc/1.5 M Na_2CO_3; (c) NaOMe, MeOH.



11 XAr = O-(2-naphthyl)
10 XAr = S-(2-naphthyl)
15 XAr = O-(2-naphthyl)
12 XAr = S-(2-naphthyl)

Scheme 2 Synthesis of 6-thio derivatives **29**, **15** and **12**. Reagents and conditions: (a) *p*-toluenesulfonyl chloride, pyridine, 16 h, 0°C; (b) acetic anhydride, pyridine, 16 h, rt; (c) potassium thioacetate, *N*, *N*-dimethylformamide, 16 h, 80°C; (d) KOH, MeOH, 1 h, rt.

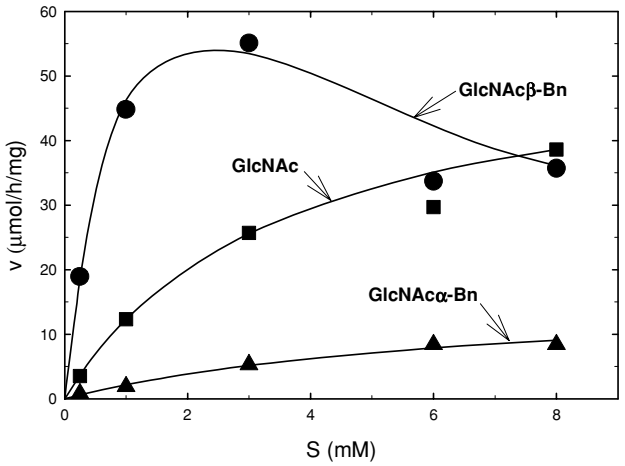


Fig. 1 Galactosyl transfer as a function of GlcNAc, GlcNAcα-Bn and GlcNAcβ-Bn substrate concentrations. The curves were fitted by non-linear regression with best fit used to determine kinetic mechanism (Table 1).

Table 2 Substrate specificity of β4Gal-transferase: Role of the glycosidic linkage of GlcNAc-terminating substrates

Compound		
No.	Structure	Activity %
1	GlcNAcβ-Bn	100
2	GlcNAc	29
3	GlcNAcα-Bn	6
4	GlcNAcβ-(*p*-NO_2Ph)	44
5	GlcNAcα-(*p*-NO_2Ph)	4
6	1-DeoxyGlcNAcβ-[(*n*-hexylamino)methyl][a]	126
7	1-ThioGlcNAcβ-Et[a]	127
8	GlcNAcβ-(2-naphthyl)[a]	<1
9	1-ThioGlcNAcβ-(2-naphthyl)[a]	<2
10	1-Thio-*N*-butyrylGlcNβ-(2-naphthyl)[a]	<1
11	*N*-ButyrylGlcNβ-(2-naphthyl)[a]	<1
12	1,6-Dithio-*N*-butyrylGlcNβ-(2-naphthyl)[a]	<1

Compounds **1–12** were used as substrates in β4GalT assays (at a concentration of 2 mM), as described in the Methods section.
[a]Assays were carried out in 10% methanol. The activities are given relative to the activity of control substrate **1** at the same concentration in the same solvent matrix. The activity using control substrate was 10 to 40 μmol/h/mg protein.

acceptors having hydrophobic aglycone groups are shown in Table 1, and the relative rates of galactosyl transfer to a number of compounds at either 1 mM or 2 mM concentrations are shown in Table 2 and Table 3. All of these data demonstrate the relative importance of the hydrophobic aglycone group in binding to the active site of the enzyme, although some of the substrates with K_m values in the range of 0.1–0.5 mM, namely, compounds **19** and **24**–**28**, have relatively lower values for V_{max}, suggesting a slower rate of release of products from the active-site cleft.

It is noteworthy that the benzyl glycoside **1** and the corresponding phenyl glycoside **13** exhibited approximately equal

Table 3 Substrate specificity of β4Gal-transferase: Role of the aglycone group of GlcNAc-terminating substrates

Compound		
No.	Structure	Activity %
1	GlcNAcβ-Bn	100
13	GlcNAcβ-Ph	104
14	1-ThioGlcNAcβ-Ph	86
15	6-Thio-*N*-butyrylGlcNβ-(2-naphthyl)[b]	<4
16	GlcNAcβ-(*p*-NO_2Bn)[a]	51
17	GlcNAcβ-(*p*-MeOBn)[c]	97
18	GlcNAcα-(11-phenoxyundecyl)[a]	<1
19	GlcNAcβ-(11-phenoxyundecyl)[a]	8
20	GlcNAc(β1-3)GalNAcα-(*p*-NO_2Ph)	72
21	GlcNAc(β1-6)[GlcNAc(β1-3)]GalNAcα-Bn	101
22	GlcNAc(β1-6)GalNAcα-Bn	104
23	GlcNAc(β1-2)Man(α1-6)Glcβ-(*p*-NO_2Ph)	61
24	GlcNAcβ-(3-isoquinolinyl)[a]	80
25	GlcNAcβ-(6-quinolinyl)[a]	62
26	GlcNAcβ-(8-quinolinyl)[d]	106
27	GlcNAcβ-[8-(5-chloroquinolinyl)][d]	51
28	1-ThioGlcNAcβ-(2-quinolinyl)[d]	53

Compounds **13–28** were used as substrates in β4GalT assays (at a concentration of 1 mM), as described in the Methods section.
[a]Assays were carried out in 10% methanol.
[b]Assays were in 3% methanol and 2% DMSO.
[c]Assays were in 5% methanol.
[d]Assays were carried out in 7% isopropanol. The activities are given relative to the activity of control substrate **1** at the same concentration in the same solvent matrix. The activity using control substrate was 10–40 μmol/h/mg protein.

activity at 1 mM substrate concentration, but the phenyl derivative had a K_m value 1.7-fold lower than that of **1** and a V_{max} 2.6-fold lower than that of **1**. However, the *p*-nitrophenyl glycoside **4** exhibited activity that was only 44% of **1** at 2 mM substrate concentration (Table 2) and was 9-fold lower with respect to V_{max} (Table 1). In the case of the glycoside GlcNAcβ-(*p*-MeOBn) (**17**), having an electron-donating group, the activity was equal to that of the control substrate **1** at 1 mM, while the corresponding *p*-nitrobenzyl glycoside **16** showed 51% of the activity of **1** (Table 3). Moreover, the rate of galactosyl transfer was 2.4-fold higher in the case of the phenyl glycoside **13** relative to the corresponding *p*-nitrophenyl glycoside **4** (Tables 2 and 3). These data suggest that the presence of the electron-withdrawing nitro group results in a lower overall turnover rate at the active site.

Table 3 shows a number of glycosides of GlcNAc having relatively large hydrophobic aglycone groups. For example, the α-glycoside **18** and the β-glycoside **19** each contain a phenoxyundecyl group as the aglycone, and, significantly, the former glycoside did not support β4GalT activity, and the galactosyl transfer activity of the latter was only 8% of that of the control **1**, suggesting that GlcNAc residues having long flexible hydrophobic aglycone chains are poor β4GalT sub-

Table 4 Inhibition of β4Gal-transferase by glucosamine analogs

Compound		Inhibitor (mM)	Inhibition (%)
8	GlcNAcβ-(2-naphthyl)	0.2	28
		0.4	72
		1.0	92
9	1-ThioGlcNAcβ-(2-naphthyl)	0.2	59
		0.4	77
		1.0	91
10	1-Thio-*N*-butyrylGlcNβ-(2-naphthyl)	0.2	85
		0.4	98
		1.0	100
11	*N*-ButyrylGlcNβ-(2-naphthyl)	0.2	31
		0.4	72
		1.0	87
12	1,6-Dithio-*N*-butyrylGlcNβ-(2-naphthyl)	0.2	3
		0.4	22
		1.0	45
15	6-Thio-*N*-butyrylGlcNβ-(2-naphthyl)	1.0	19

Compounds **8–12** and **15** were assayed as inhibitors of β4Gal-transferase at various concentrations using GlcNAcβ-Bn **(1)** as the substrate (at a concentration of 1 mM), as described in the Methods section.

strates. Glycosides having hydrophobic 2-naphthyl groups (compounds **8**, **9**, **10**, **11, 12** and **15**) also were not substrates (Tables 2 and 3); however, some of these were found to be potent inhibitors of the galactosyltransferase, demonstrating that they did bind to the enzyme (Table 4 and below). In contrast to the 2-naphthyl glycosides, a series of isoquinolinyl (see **24**) and quinolinyl glycosides (see **25**–**28**) were substrates for β4GalT (Table 3); the synthesis of these novel compounds is described in this article. Presumably, since the sizes of the naphthyl, isoquinolinyl and quinolinyl groups are similar, the difference in behaviour of the naphthyl glycosides and those having the nitrogen-containing heterocycles as aglycones can be attributed to the differences in electronic structures.

The kinetic efficiencies of compounds GlcNAcβ-(6-quinolinyl) (**25**) and 1-thioGlcNAcβ-(8-quinolinyl) (**28**) were comparable to that of **1**. Although the K_m values of the quinolinyl derivatives were relatively low, ranging from 0.09 to 0.44 mM (Table 1), showing that they bind well to the enzyme, the V_{max} values were also relatively low, possibly indicating that the products bind to the enzyme and are not readily released, resulting in slow turnover numbers. These results demonstrate again that the bulkiness of the naphthyl aglycone is not the factor preventing binding to the enzyme in a favourable configuration for activity. However, it appears that the position of the ring-nitrogen atom, the presence of a substituent such as a chloro group, the linkage position on the heterocyclic ring and the nature of the linking atom all have considerable influence on the formation of a productive complex and rate of galactosyl transfer.

Benzyl and *p*-nitrophenyl glycosides of di- and trisaccharides having GlcNAc at the non-reducing end (compounds **20**–**23**) were also tested as substrates to determine whether underlying sugar structures had any effects on enzyme activity. There was no evidence of significant differences in the rates of transfer induced by the insertion of additional sugar residues in the substrates (Table 3). With the limited number of these acceptors tested, in each case there was no significant difference in recognition of the sugar (GalNAc or Man) attached to the terminal GlcNAc in the oligosaccharide. Thus, at least when small soluble oligosaccharides are used as acceptors, recognition of the aglycone group at the active-site binding pocket of the enzyme does not appear to extend very far beyond the anomeric carbon bearing the aglycone.

Glycopeptides as substrates

Glycopeptides are natural acceptors for β4GalT; we therefore wanted to study the effects of the (glyco)peptide aglycone groups in β4GalT substrates. Therefore, a series of GlcNAc-terminating *O*-glycosylated decapeptides having sequence similarity to MUC 2 mucin tandem repeat sequences was studied. The glycopeptides had been synthesized on identical peptide backbones containing six Thr residues, and each glycopeptide contained one *O*-glycan chain. The glycans had terminal GlcNAc residues of core 3 (GlcNAcβ1-3GalNAcα), core 4 (GlcNAcβ1-6[GlcNAc-β1-3] GalNAcα) or core 6 (GlcNAcβ1-6GalNAcα) structures, and were linked to different Thr residues of the decapeptide backbone. Table 5 shows the activity observed with each glycopeptide as a substrate at 0.5 mM concentration, relative to the control substrate GlcNAcβ-Bn (**1**). Although less active than **1**, all of the glycopeptides were significant substrates for β4GalT, with those having two GlcNAc residues (core 4) in the molecule being the best substrates of this series.

The rates of galactosyl transfer, however, were found to vary within the family of isomeric glycopeptides, indicating that the GlcNAc residues in different positions of the peptide were not equivalent as acceptors. In the series of glycopeptide substrates containing core 3, optimal activity was seen when the core 3 structure was attached to Thr5 (see **GP3**). By comparison, the core 6 glycopeptide series showed optimal activity when the core 6 structure was attached to Thr2 (see **GP13**). Reverse-phase HPLC analysis of assays with **GP7**, **GP8** and **GP9** substrates, each of which has two GlcNAc residues (core 4) attached to various sites in the peptide, showed that in all cases one major radioactive product peak was found. Assays with **GP9** as the substrate yielded a gradual increase from 18 to 46% conversion of substrate into

Table 5 Substrate specificity of β4Gal-transferase towards GlcNAc-terminating O-glycopeptides

Compound		
No.	Structure	Activity %
1	GlcNAcβ-Bn	100
GP1	TTTVTPTP (GlcNAcβ3GalNAcα)**T**G	14
GP2	TTTVTP (GlcNAcβ3GalNAcα)**T**PTG	30
GP3	TTTV (GlcNAcβ3GalNAcα)**T**PTPTG	65
GP4	TT (GlcNAcβ3GalNAcα)**T**VTPTPTG	12
GP5	T (GlcNAcβ3GalNAcα)**T**TVTPTPTG	21
GP6	TTTVTPTP(GlcNAcβ6[GlcNAcβ3] GalNAcα)**T**G	69
GP7	TTTV(GlcNAcβ6[GlcNAcβ3]GalNAcα) **T**PTPTG	86
GP8	TT(GlcNAcβ6[GlcNAcβ3]GalNAcα) **T**VTPTPTG	64
GP9	T(GlcNAcβ6[GlcNAcβ3]GalNAcα) **T**TVTPTPTG	96
GP10	TTTVTP(GlcNAcβ6GalNAcα)**T**PTG	36
GP11	TTTV(GlcNAcβ6GalNAcα)**T**PTPTG	36
GP12	TT(GlcNAcβ6GalNAcα)**T**VTPTPTG	18
GP13	T(GlcNAcβ6GalNAcα)**T**TVTPTPTG	54

Compounds **GP1–13** were used as substrates in β4GalT assays at a concentration of 0.5 mM, as described in the Methods section. The activities are given relative to the activity of control substrate **1** at the same concentration in the same solvent matrix. The highlighted Thr residues represent the *O*-glycosylation sites.

product in 30-, 60-, 90-and 120-min incubation times. With **GP8** as the substrate, the conversion into product was 16 and 18% after 30- and 60-min incubation times, respectively.

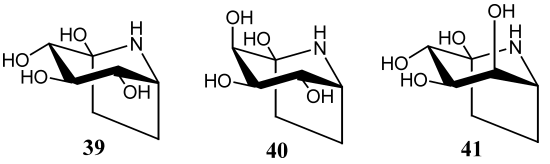
Roles of the sugar ring and substituents

In order to define further the sugar structure required as an acceptor for galactosyl transfer, a number of different analogs of GlcNAc were tested as substrates (Table 6). Also shown in Table 6 are three compounds, namely, calystegine B2 (**39**), calystegine B3 (**40**) and calystegine B4 (**41**) which are bicyclic sugar analogs having nitrogen in the ring; these compounds were not substrates for β4GalT. Compound **42** [1,5-dideoxy-5-(*n*-hexylamino)GlcNAc*p*] also has a nitrogen in the ring, and no activity was observed. None of the C4-modified derivatives of GlcNAc (compounds **33**–**37**) were found to be substrates, an expected result since the enzyme utilizes the C4-hydroxyl group in forming the new glycosidic linkage. The 3-deoxy derivatives **31** and **32** were not active, but 3-deoxy-3-fluoroGlcNAc (**30**) showed significant activity at 2 mM, namely, 40% of the activity of the control GlcNAcβ-Bn (**1**), and 1.4-fold higher than with GlcNAc (**2**) as the acceptor. Thus, it appears that the presence of a group at C3 is important in orientating the acceptor in the binding pocket. Compound **30** was found to have a relatively high

Table 6 Substrate specificity of β4Gal-transferase: Role of the ring substituents of GlcNAc

Compound		
No.	Structure	Activity %
1	GlcNAcβ-Bn	100
2	GlcNAc	29
29	6-ThioGlcNAcβ-Bn[a]	12
30	3-Deoxy-3-fluoroGlcNAc	40
31	3-DeoxyGlcNAcα-Bn	<1
32	3-DeoxyGlcNAc	1
33	4-DeoxyGlcNAcβ-Me	<1
34	4-DeoxyGlcNAcα-Me	<2
35	4-DeoxyGlcNAcα-Bn	<1
36	4-Deoxy-4-fluoroGlcNAcα-Bn	<1
37	3,4-DideoxyGlcNAc	<1
38	6-DeoxyGlcNAc	13
39	Calystegine B2	<1
40	Calystegine B3	<1
41	Calystegine B4	<1
42	1,5-Dideoxy-5-(*n*-hexylamino)GlcNAc*p*[a]	2

Compounds **2, 29–42** were used as substrates in β4GalT assays at a concentration of 2 mM, as described in the Methods section. [a]Assays were carried out in 10% methanol. The activities are given relative to the activity of control substrate **1** at the same concentration in the same solvent matrix.



K_m value of 10.6 mM, indicating that the C3-fluoro group does not bind with the same efficiency as the C3-hydroxyl group, but this substrate has a V_{max} equivalent to the control substrate **1** (Table 1). The results suggest that in an active substrate, the C3-hydroxyl can be replaced by a smaller and more electronegative, but weaker hydrogen-bond acceptor, group such as fluorine. As regards the role of the C6-hydroxyl group of GlcNAc, it appears that the group is not essential, but that it contributes significantly to the acceptor activity. Thus, 6-deoxyGlcNAc (**38**) exhibits only 13% of the activity of **1**, whereas GlcNAc (**2**) exhibits 29% of the activity of **1**. The replacement of the C6-hydroxyl group in the glycoside **1** by a thiol group affords a compound (**29**, 6-thioGlcNAcβ-Bn) having 12% of the activity of **1**. Compared with the hydroxyl group, the thiol group is larger, less electronegative and forms weaker hydrogen bonds, features which may be important for substrate binding.

Role of the C2-substituent

The acceptor specificity of β4GalT was further defined by an evaluation of a number of GlcNAc derivatives modified

Table 7 Role of 2-*N*-substituents of glucosamine in substrate specificity of β4Gal-transferase

Compound		
No.	Structure	Activity %
1	GlcNAcβ-Bn	100
2	GlcNAc	29
43	*N*-ButyrylGlcNβ-Bn	116
44	*N*-*p*-ToluenesulfonylGlcN	12
45	*N*-MethanesulfonylGlcN	6
46	*N*-TrifluoroacetylGlcN	39
47	*N*-TrimethylacetylGlcN	<1
48	*N*,*N*-DimethylGlcN	<1
49	*N*-IsopropylGlcN	<1
50	*N*-(4-MeOBn)GlcN	<1

Compounds **2, 43–50** were used as substrates in β4GalT assays (at a concentration of 2 mM), as described in the Methods section. The activities are given relative to the activity of control substrate **1** at the same concentration in the same solvent matrix.

at C2-nitrogen (Table 7). Compared with GlcNAc, Glc is an extremely poor substrate of the holoenzyme in the absence of lactalbumin, and glucosamine (GlcN) was not a substrate. However, the enzyme can accommodate a wide range of substituents, some quite large and bulky, at the C2 position. Thus, *N*-butyrylGlcNβ-Bn (**43**) was found to be an excellent substrate having kinetic parameters similar to those of GlcNAcβ-Bn (**1**). The kinetics with compound **43** also showed significant substrate inhibition with a K_{si} of 8.13 mM (Table 1). Replacement of the *N*-acetyl group by an *N*-trifluoroacetyl group yielded a substrate (**46**, *N*-trifluoroacetylGlcN) having 1.3-fold higher activity than GlcNAc (**2**) when assayed at 2 mM (Table 7). The enzyme can even accommodate a sulfonamidyl group at C2 as in *N*-*p*-toluenesulfonylGlcN (**44**) and *N*-methanesulfonylGlcN (**45**). However, the V_{max} values of these substrates were low with very high K_m values (Table 1). By comparison, all of the *N*-alkylated derivatives (**48–50**) of GlcN were inactive as substrates. The results suggest that an *N*-acyl group at C2 may be essential for strong substrate binding. Interestingly, *N*-trimethylacetylGlcN (**47**) was inactive, although it does possess an *N*-acyl group at C2; in this case steric hindrance by the three methyl groups may have prevented substrate binding (Table 7).

Inhibition of β4GalT

Compounds that were identified as poor substrates for the β4GalT, or else had no acceptor activity, were tested as potential inhibitors of galactosyl transfer to 1 mM GlcNAcβ-Bn (1) (Table 4). Most of the compounds shown in Tables 2, 3, and 5–7 that were found to be inactive as substrates also failed to inhibit transferase activity, indicating that they did not bind to the enzyme. However, GlcNAcβ-(2-naphthyl) (**8**) was reported [23] to be an effective inhibitor of the enzyme. The current results show that the corresponding *N*-butyryl derivative, *N*-butyrylGlcNβ-(2-naphthyl) (**11**), has inhibitory properties similar to those of **8**, and the thioglycoside analog of **8**, namely, 1-thioGlcNAcβ-(2-naphthyl) (**9**) is an even more effective inhibitor than is **11**. Since both the thioglycosidic linkage and the *N*-butyryl group appeared to be favourable modifications as regards substrate binding, while the incorporation of the 2-naphthyl group appeared to inhibit activity, we synthesized and evaluated 1-thio-*N*-butyrylGlcNβ-(2-naphthyl) (**10**) as a potential inhibitor. This compound was inactive as a substrate but was found to be a highly effective inhibitor of β4GalT. At equimolar or higher concentrations of inhibitor **10** relative to substrate **1**, the inhibitor caused 100% inhibition. The best fit for the kinetic mechanism of inhibition was uncompetitive as judged by non-linear regression analysis, with a K_i of 0.01 mM for compound **10**. When plotted on a Lineweaver–Burk plot, the data yielded a series of parallel lines, a result which is consistent with an ordered mechanism in which the nucleotide sugar binds the enzyme prior to acceptor binding. Compound **9** showed similar uncompetitive inhibition with a K_i of 0.06 mM.

The 6-thio analog of **10**, namely, 1,6-dithio-*N*-butyrylGlcNβ-(2-naphthyl) (**12**), was less effective as an inhibitor compared to **10** (45% inhibition at equimolar concentrations with substrate **1**), confirming that the C6-hydroxyl group is important for binding to the enzyme. Analogously, replacement of the C6-hydroxyl in *N*-butyrylGlcNβ-(2-naphthyl) (**11**) by a thio group to afford 6-thio-*N*-butyrylGlcNβ-(2-naphthyl) (**15**) drastically decreased the ability of the thio compound to inhibit the enzyme (Table 4). A comparison of the inhibition of β4GalT by thioglycosides **9** and **10** to that of the corresponding oxygen analogs **8** and **11** suggested that the two types of glycosides were essentially equivalent in their abilities to bind to and inhibit the enzyme.

Discussion

Better insight into the geometry of the active site of β4GalT could lead to the development of therapeutic enzyme inhibitors that could control inflammatory disease and other pathological processes. Therefore, the goal of this work was to obtain a better understanding of the requirements for substrate binding and inhibition of β4GalT. For these studies, several series of acceptor substrate analogs were studied to determine the molecular parameters required for substrate and inhibitor binding to β4GalT.

Our results demonstrate that the aglycone group plays a significant role in substrate binding and acceptor activity. In this work it was found that the β-anomeric configuration of GlcNAc substrates was important; the α-anomers were poor substrates, probably owing to the steric hindrance of an

axially oriented aglycone group. However, transfer of Gal by β4GalT to UDP-α-GlcNAc *in vitro* has been reported [49].

Kajihara et al. [22] demonstrated that the ring oxygen of GlcNAc was not essential for β4GalT activity and could be replaced by carbon. In the present study, the results obtained with the limited number of compounds in which the ring oxygen has been replaced by nitrogen suggests that this structural modification is detrimental as regards activity. This observation is consistent with studies of lactose synthase which acts poorly on 1-deoxynojirimycin, a compound containing nitrogen in the ring [19]. However, lactose synthase has significant activity in the presence of α-lactalbumin towards 5-thioglucose, a compound containing sulfur in the ring [19].

It has been recently shown [50] that a series of *N*-acylglucosamines having acyl chain lengths up to 7 carbons were good substrates for β4GalT. The exception was *N*-benzoylGlcN which was a poor substrate. These results are consistent with our observations, namely, that the presence of an *N*-acyl group at C2 is an essential structural feature for high activity.

Carbohydrates attached to 1–3 or 1–6 linked branches are expected to differ in their three-dimensional presentation, and β4GalT has been shown to distinguish between these two branches. From the results in Table 3, a clearly defined branch specificity towards GlcNAc substrates in 1–3 or 1–6 linkages in relatively small *O*-glycan core structures was not evident. Glycopeptides containing *O*-glycan chains having terminal non-reducing GlcNAc residues are good substrates, however the more natural glycopeptides are not better acceptors than is GlcNAc linked to an aromatic benzyl group, as in compound **1**. Although the enzyme acts at a distance of at least two sugar residues away from the peptide chain, it still differentiates between the positions of GlcNAc residues within the peptide chain. This finding extends our previous observation of a site-directed *O*-glycosylation. Thus, the enzymes synthesizing *O*-glycan cores 1 and 3 [51–53], as well as the β4GalT extending core structures, are all influenced by the peptide structure adjacent to the *O*-glycosylation site. This effect may be based on different conformations of peptide, and conformations or exposure of carbohydrates at these sites. *O*-Glycosylated mucin-type glycopeptides containing Pro residues often have an extended conformation with specific orientation of the glycan chain [54]. Depending on the position of the glycan chain, the peptide may interact with specific amino acids of the extended substrate binding site of the enzyme.

Our results show that an extension of the glycan chain of the acceptor substrate had a minor impact on enzyme activity. This observation is consistent with those by Ujita et al. [55] who found that longer oligosaccharide chains as acceptor substrates exhibited similar kinetic parameters to those of trisaccharide substrates, and oligosaccharides with both O-glycan core 3 and core 4 structures had similar K_m values. Ramasamy *et al.* [56] found that the presence of a less restrained 1–6 linkage, compared to a 1–3 linkage, in a trisaccharide acceptor substrate reduced the K_m value 10-fold.

Naphthyl derivatives clearly are not substrates for β4GalT. We have demonstrated that this lack of substrate activity of the naphthyl derivatives is not simply attributable to steric factors, since the similar bulky quinolinyl and isoquinolinyl derivatives are supporting the activity. Thus, the enzyme–acceptor-substrate binding site is large enough to accommodate these bulky substrates. It is possible that the more hydrophobic naphthyl group binds significantly to the enzyme whereas the quinolinyl and isoquinolinyl groups bind but are released and allow the enzyme to bind more acceptor substrate. In contrast to naphthyl groups, quinolinyl groups appear to have favourable chemical or electronic properties which support activity. Ramakrishnan *et al*. [10] showed that the acceptor binding site of β4GalT involves Tyr, Phe and Trp residues [57] which potentially could interact with an aromatic aglycone group. It is thus possible that the naphthyl group binds tightly to these hydrophobic amino acids in the substrate binding site, thereby preventing catalysis or the release of enzyme product. In contrast, quinolinyl and isoquinolinyl compounds may not bind as tightly to hydrophobic amino acids. Because of their dipolar character induced by the presence of a nitrogen atom, these compounds may instead associate with charged amino acids. A crystal structure of β4GalT together with inhibitor may reveal how these compounds bind to the enzyme.

The glycosidic naphthyl GlcNAc inhibitors are relatively small and hydrophobic, having the potential to cross cell membranes. Therefore, these compounds are ideal for the development of agents that modify galactosylation in cells or tissues. It remains to be shown if the inhibitors also act on other Gal-transferases that utilize GlcNAc as the substrate, and on other glycosyltransferases.

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