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Synthesis of uridine-5-propylamine derivatives and their use in affinity chromatography of *N*-acetylglucosaminyltransferases I and II

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Abstract

The C-5 substituted uridine derivatives UDP-5-propylamine (**7**) and UDP-GlcNAc-5-propylamine (**8**) were synthesized in good yields by Heck alkylation of the 5-mercuriuridines, followed by hydrogenation. The products were characterized by ^{1}H and ^{13}C NMR spectroscopy, electrospray mass spectrometry and UV spectrophotometry. The amines are of interest for the preparation of affinity probes for glycosyltransferases. The benzoylbenzamides of **7** and **8** show strong competitive inhibition of *N*-acetylglucosaminyltransferases I and II with K_i values ranging from 30 to 100 μM (without irradiation) and may be useful as active site-directed photoaffinity labels. A conjugate of **8** and Sepharose was used for affinity chromatographic purification of *N*-acetylglucosaminyltransferases I and II. The results indicate that this affinity gel is a stable alternative to the commonly used but unstable UDP-GlcNAc-5-Hg-thiopropyl conjugate.

Keywords: Uridine-5-propylamine derivatives; *N*-Acetylglucosaminyltransferases I and II; Affinity chromatography

1. Introduction

Affinity chromatographic methods, employing immobilized substrate or product derivatives, have been widely used for the purification of natural [1] and recombinant [2,3] glycosyltransferases. *N*-Acetylglucosaminyltransferases I–VI (GlcNAc-T I–VI)

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control the branching pattern of *N*-glycans in the biosynthesis of *N*-glycoproteins [4–6] and catalyse the general reaction

$$\text{UDP-}\alpha\text{GlcNAc} + \text{R-OH} \rightarrow \text{UDP} + \text{R-O-}\beta\text{GlcNAc}$$

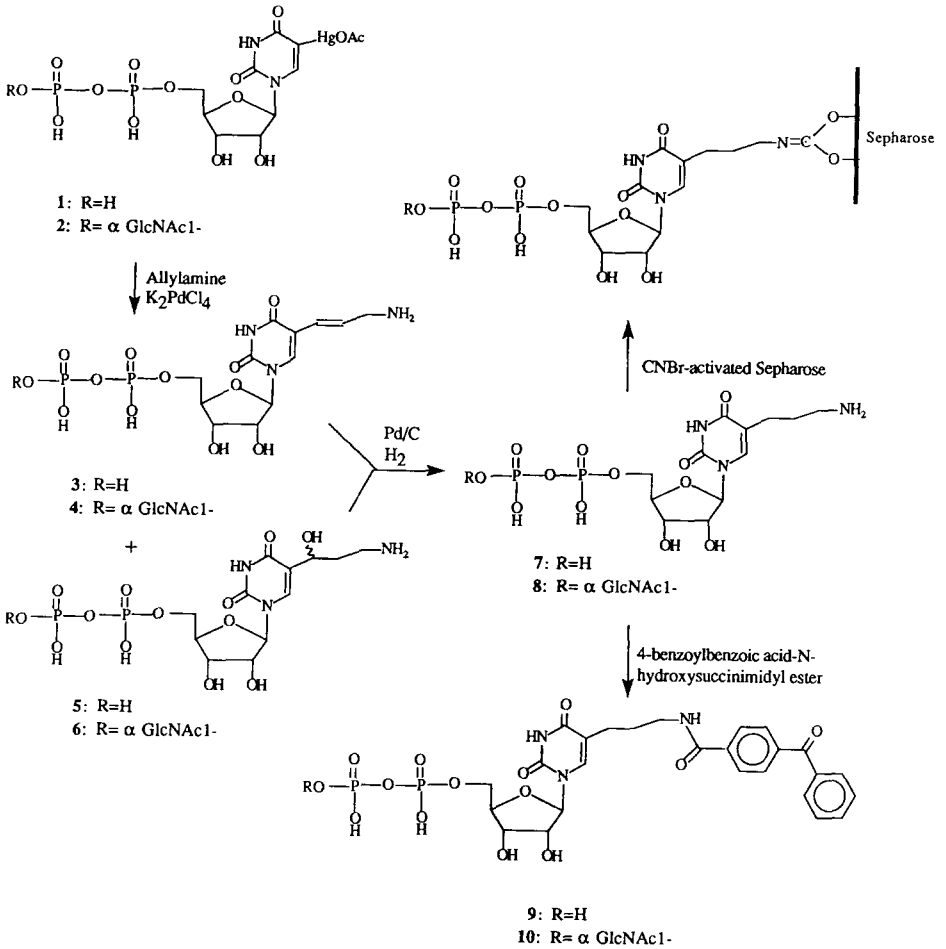
with R being a specific oligosaccharide. GlcNAc-T I [7], II [8], III [9], and V [10,11] have been purified with UDP- or UDP-GlcNAc-agarose conjugates to near homogeneity. In most cases, uridine-hexanolamine pyrophosphate diesters (UDP-hexanolamine) were coupled via the amino group to cyanogen bromide-activated Sepharose, resulting in stable and reusable conjugates for many applications. Oligosaccharide acceptor substrate conjugates have also been used in some cases [7,9–11], but the oligosaccharide ligands are difficult to synthesize or have to be isolated from natural sources.

Some enzymes such as UDP-GlcNAc:Manα1-6R β1-2-*N*-acetylglucosaminyltransferase II (EC 2.4.1.143, GlcNAc-T II) do not bind to the UDP-hexanolamine-agarose conjugate [8]. Bendiak and Schachter [12] found that C-5-substituted uridine derivatives show increased binding to GlcNAc-T II compared with other uridine derivatives. Consequently, a UDP-5-Hg-S-$CH_2CHOHCH_2$-O-Sepharose conjugate and the corresponding UDP-GlcNAc derivative were found to bind to both GlcNAc-T II and GlcNAc-T I and were used in the purification of both enzymes [7,8]. However, wider application of this conjugate for the small- and large-scale purification of natural and recombinant glycosyltransferases was hindered by its instability. The mercury–carbon bond is extremely sensitive to cleavage by electrophiles and reducing agents (such as hydroquinones) [13] and is slowly oxidized by air [14]. In addition, the thiopropyl-Sepharose ligand is readily substituted by other thiols such as β-mercaptoethanol or exposed thiol groups on proteins [13]. Therefore, the C-5-mercuri conjugates are not stable for most applications and have to be prepared fresh for each run in order to obtain reproducible results [8]. Further, protein purification yields may be low if the protein has a thiol group which is exposed or near the active site.

It is therefore of interest to synthesize, for use in affinity chromatography, stable uridine-C-5-substituted agarose conjugates which can be used repeatedly and do not react covalently with proteins. We now report on the synthesis of new uridine-5-propylamine derivatives and affinity probes. We also demonstrate the use of Sepharose conjugates of these affinity probes for the purification of GlcNAc-T I and GlcNAc-T II.

2. Results and discussion

Synthesis of C-5-substituted uridine derivatives.—Stable C-5-substituted uridine derivatives can be obtained by alkylation of C-5-mercurated uridines with olefins and Group VIII metal salts, according to Heck [15–17], a reaction which has been studied in detail by Bergstrom and Ogawa [18]. The mechanism probably involves an exchange of the uridine ligand from the organomercury compound to a palladium–olefin complex (formed in situ), formation of a C–C bond by *cis* insertion, and reductive elimination of Pd^0 and HCl. Non-conjugated 1-olefins react normally to give a mixture of the 1-uridine-substituted olefins and 1-uridine-2-methoxyalkanes as the main products when the reaction is performed in methanol. Upon hydrogenation of the mixture, homoge-



Scheme 1.

neous C-5 alkyl derivatives are obtained in good yields [18]. Langer et al. [19] employed the Heck alkylation for the synthesis of C-5 allylamine derivatives of dUTP and UTP, which were purified from a mixture of unidentified byproducts. Meikle et al. [20] reported on the synthesis of the corresponding C-5 allylamine derivatives of UDP and UDP-Glc and did not observe formation of byproducts.

In order to test if C-5 allylamine derivatives of uridine are useful for the preparation of affinity gels, we repeated the synthesis of UDP-5-allylamine (**3**) (Scheme 1) reported by Meikle et al. [20]. Mercuration of UDP with mercuric acetate gave UDP-5-mercuric acetate (**1**) [13], and **1** was converted with allylamine and potassium tetrachloropalladate (II). The product was purified by anion-exchange chromatography and UV analysis showed two maxima at λ_{max} 243 and 289 nm, as previously reported, indicating the presence of an exocyclic double bond. However, ^{1}H NMR analysis showed that a

mixture of three compounds in a molar ratio of 10:3:3 was obtained; this mixture could not be separated by anion-exchange chromatography, gel filtration or TLC on silica gel.

The mixture was analysed by ^{1}H and ^{13}C NMR spectroscopy and assignments were possible with the help of homo- and hetero-nuclear correlation experiments and attached proton tests. The three compounds were clearly C-5-substituted UDP derivatives, but differences were observed in the signals for the substituent and uridyl H-6. The ^{1}H NMR data for the main product show two CH groups with a doublet at 6.58 ppm and a double triplet at 6.50 ppm, and a CH_2X group with a doublet at 3.74 ppm. The data are in agreement with the structure given for the allylamine derivative **3**. The large vicinal coupling constant of 16 Hz between the olefin protons proves the *trans* configuration of the double bond. The ^{1}H NMR data of the minor products show a CHX, a CH_2, and a CH_2Y group by multiplets at 4.80, 2.31–2.11, and 3.16 ppm respectively, with the signal for the CH_2 group being resolved into two multiplets. The ^{13}C NMR data for the minor products are almost identical with each other but show small differences in chemical shifts. The data are in agreement with the diastereomeric structure UDP-5-$CHXCH_2CH_2Y$.

Final proof of the given structures for the major product **3** and the minor products **5** (Scheme 1) was provided by electrospray mass spectrometry analysis, which shows m/z 458.2 ($M-H^-$ for **3**, +0.16, 100%) and 476.2 ($M-H^-$ for **5**, +0.15, 73%). The diastereomeric aminopropanols **5** probably arise from attack by the solvent water on a palladium alkyl intermediate and replacement of palladium by OH^-. A similar mechanism has been proposed by Bergstrom and Ogawa [18], based on mechanistic studies, for the formation of the corresponding α-methoxy derivatives when the reaction was performed in methanol. The formation of **5** was not observed by Meikle et al. [20]; however, the authors did not present NMR data for **3**. They reported uridyl H-6 at 7.72 ppm for a C-5-allylamide derivative of UDP-Glc and assigned a signal at 7.61 ppm to allylic NH. The latter signal is, however, more likely to arise from H-6 of a second compound, since the NH group is expected to exchange with deuterium in deuterium oxide and is not detected in a routine ^{1}H NMR experiment.

The mixture of **3** and **5** was subjected to hydrogenolysis on Pd/C, upon which the exocyclic double bond in **3** was reduced and concomitantly the C–O bond of the mesomeric stabilized allyl alcohol moiety in **5** was reductively cleaved [21] to give almost quantitatively UDP-propylamine (**7**) in an overall yield of 39% starting from UDP. The reaction was monitored by ^{1}H NMR, which shows that **3** is reduced more rapidly than **5**. The structure of **7** (Scheme 1) was confirmed by ^{1}H NMR and electrospray MS.

UDP-GlcNAc-5-propylamine (**8**) was synthesized in a similar way. Mercuration of UDP-GlcNAc gave UDP-GlcNAc-5-HgOAc (**2**) [8], which was alkylated with allylamine and potassium tetrachloropalladate(II). After ion-exchange chromatography, a mixture of products was obtained which was analysed by ^{1}H NMR and electrospray MS and found to consist of the C-5-allylamine substituted compound **4** and the diastereomeric aminopropanols **6** in a molar ratio of 5:2, with **6** being a 1:1 mixture of two diastereomers. Compounds **4** and **6** were hydrogenolysed on Pd/C to give UDP-GlcNAc-5-propylamine (**8**) in an overall yield of 36% starting from UDP-GlcNAc. Compound **4** reacts more rapidly than **6** and the reaction may be monitored by TLC,

Table 1
Kinetic parameters for GlcNAc-T I and GlcNAc-T II with uridine derivatives as substrates and inhibitors

Uridine derivative	GlcNAc-T I		GlcNAc-T II	
	K_M [a] (μM)	K_i [a] (μM)	K_M [a] (μM)	K_i [a] (μM)
UDP-GlcNAc	190		720	
UDP-GlcNAc-5-propylamine (**8**)		ND [b]		ND [b]
UDP-GlcNAc-5-propyl-BBA [c] (**10**)		100		55
UDP		44 [d]		980 [e]
UDP-5-propylamine (**7**)		ND [b]		ND [b]
UDP-5-propyl-BBA [c] (**9**)		30		I [f]

[a] K_M and K_i values were determined from enzyme assays at four or more donor substrate concentrations by linear double-reciprocal Lineweaver–Burk plots. The standard deviations of the slopes and intercepts ranged from 2 to 6% and 6 to 20% for K_M and K_i, respectively. The R^2 values of the regressions ranged from 0.98 to 1.00.
[b] No Inhibition detected when assayed with 0.7 mM uridine derivative.
[c] BBA = benzoylbenzamide.
[d] K_i obtained with varying concentrations of UDP-GlcNAc and a fixed concentration of acceptor [7].
[e] K_i obtained with varying concentrations of UDP-GlcNAc and a fixed concentration of acceptor [12].
[f] Inhibition detected, but Lineweaver–Burk plots were not linear.

since **6** and **8** show slightly different mobilities. ^{1}H NMR and electrospray MS data confirm the structure given for **8** (Scheme 1).

The amines **7** and **8** are new C-5-substituted nucleotide derivatives which can be selectively modified at the amino group for the preparation of various affinity probes, e.g., they can be coupled to a matrix for affinity chromatography. Enzyme assays using the free amines **7** and **8** as substrates or inhibitors may not be useful to predict binding of enzyme to affinity gels, since the positive charge of the amine group at physiological pH may prevent binding. We therefore synthesized the 4-benzoylbenzamides **9** and **10** to study binding to enzyme. 4-Benzoylbenzoic acid was converted into the succinimidyl ester [22] and coupled with **7** and **8** to give **9** and **10** in high yields. The benzophenone moiety is known to be a very efficient photoactivatable reactive group [23], and compounds **9** and **10** are therefore of interest as potential photoaffinity probes.

Binding studies with GlcNAc-T I and II and uridine-5-propyl derivatives.—The free amines UDP-5-propylamine (**7**) and UDP-GlcNAc-5-propylamine (**8**) do not bind to GlcNAc-T I or GlcNAc-T II, since no inhibition is detected with these compounds (Table 1), probably owing to the presence of a positively charged amino group at the assay pH. Similarly, it has been reported that UDP-hexanolamine does not inhibit GlcNAc-T I, although the enzyme is strongly bound to a conjugate of UDP-hexanolamine and Sepharose [7]. The 4-benzoylbenzamides **9** and **10** show strong competitive inhibition of GlcNAc-T I and GlcNAc-T II (Table 1 and Figs 1 and 2), with K_i values ranging from 30 to 100 μM [without UV irradiation; upon UV irradiation at 350 nm, additional inhibition is observed (data not shown)]. The corresponding iodoacetamides of **7** and **8** also showed strong inhibition of both enzymes (data not shown). Thus, conjugates of the amines **7** and **8** should bind to GlcNAc-T I and GlcNAc-T II with relatively high affinities. However, the binding constants for the Sepharose conjugates might differ from the values for the more hydrophobic conjugates **9** and **10**.

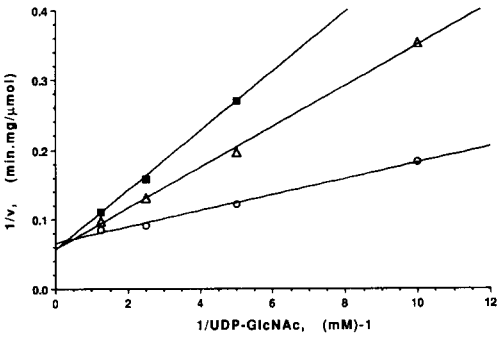


Fig. 1. Effect of compound **10** on recombinant rabbit GlcNAc-T I activity. GlcNAc-T I (0.02 mU, 2.1 ng) was assayed at several concentrations of UDP-GlcNAc, both in the absence of **10** (○) and at 80 μM (△) and 160 μM (■) concentrations of **10**. Incubations were carried out with 2 mM acceptor M_3-octyl at pH 6.1, 37°C, for 60 min. Reciprocal $1/v$ vs. $1/S$ plots indicate competitive inhibition by **10**.

Affinity chromatography of GlcNAc-T I and GlcNAc-T II.—UDP-5-propylamine (**7**) and UDP-GlcNAc-5-propylamine (**8**) were coupled to Sepharose by the cyanogen bromide method [24] to give a ligand concentration of 6.5–13 μmol/mL gel. Cyanogen bromide coupling of amines to polysaccharides has been shown to yield highly stable conjugates [25,26], probably via the formation of imino carbonic acid diesters [24]. GlcNAc-T I and II are not retarded on Sepharose alone [7,8]. Recombinant rabbit GlcNAc-T I, expressed in the baculovirus/Sf9 insect cell system [3], and purified on a UDP-hexanolamine-Sepharose column to near homogeneity with a specific activity of ca. 10–20 μmol/min/mg, was applied on a column of UDP-GlcNAc-5-propylamine (**8**)-Sepharose conjugate. The elution profile is shown in Fig. 3. Enzyme is strongly bound to the column and is not eluted with 0.1 M sodium chloride. Enzyme can be eluted slowly with 0.5 M sodium chloride and as a sharp peak with 5 mM UDP-GlcNAc at low salt concentration with about 70% recovery of the applied activity. SDS-PAGE

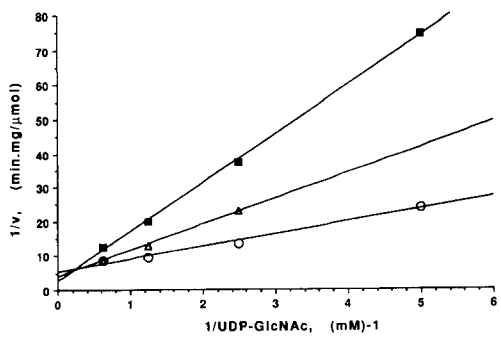


Fig. 2. Effect of compound **10** on recombinant human GlcNAc-T II activity. GlcNAc-T II (0.023 mU, 115 ng) was assayed at several concentrations of UDP-GlcNAc, both in the absence of **10** (○) and at 80 μM (△) and 200 μM (■) concentrations of **10**. Incubations were carried out with 0.4 mM acceptor GnM_3-octyl at pH 6.8, 37°C, for 60 min. Reciprocal $1/v$ vs. $1/S$ plots indicate competitive inhibition by **10**.

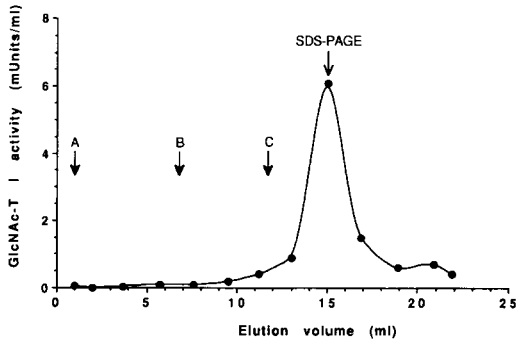


Fig. 3. Chromatography of recombinant rabbit GlcNAc-T I on UDP-GlcNAc-5-propyl GlcNAc-5-propylamine (**8**)-Sepharose. GlcNAc-T I was applied to the column in load buffer as described under Experimental. The column was washed with the load buffer containing 0.1 M sodium chloride (arrow A), followed by load buffer containing 0.5 M sodium chloride (arrow B). GlcNAc-T I was eluted with load buffer containing 0.1 M sodium chloride and 5 mM UDP-GlcNAc. Fractions were monitored for GlcNAc-T I activity and SDS-PAGE analysis was performed as indicated by the arrow.

was performed on the eluate (fraction indicated by the arrow in Fig. 3) and revealed that this step removed two high molecular mass bands (Fig. 4).

Recombinant human GlcNAc-T II, expressed in the baculovirus/Sf9 insect cell system [27a], has previously been purified to near homogeneity using UDP-5-propylamine (**7**) coupled to Sepharose in the last step of the purification with a purification factor of at least 100-fold for this step [27b]. However, retardation of GlcNAc-T II on the column seemed to decrease after a few runs and we found that UDP, and probably also the UDP-ligand, are not stable with our relatively crude recombinant GlcNAc-T II preparations [as observed by TLC (data not shown)]. A conjugate of UDP-GlcNAc-5-

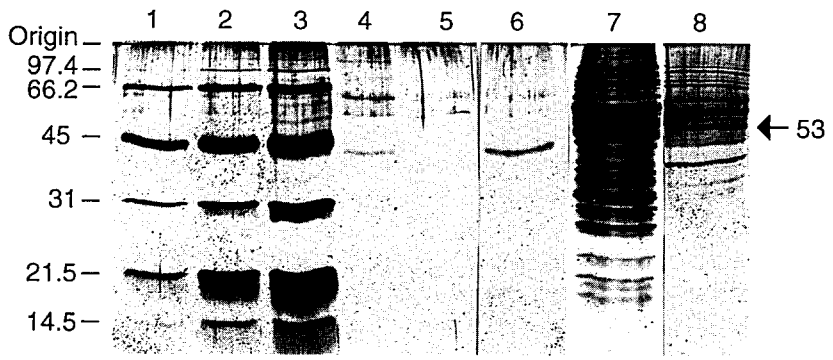


Fig. 4. SDS-PAGE analysis of GlcNAc-T I and II before and after chromatography on UDP-GlcNAc-5-propylamine (**8**)-Sepharose. Gels (12.5% polyacrylamide) were run according to Laemmli [28] in the presence of mercaptoethanol and stained by silver staining. Lanes 1, 2, and 3 contained standard proteins (40, 80, and 160 ng for each protein in each sample, respectively). Lane 5 is a buffer blank. Lanes 4 and 6 contained 2 mU GlcNAc-T I before (lane 4) and after chromatography (lane 6). Lanes 7 and 8 contained 4 mU of GlcNAc-T II before (lane 7) and after chromatography (lane 8).

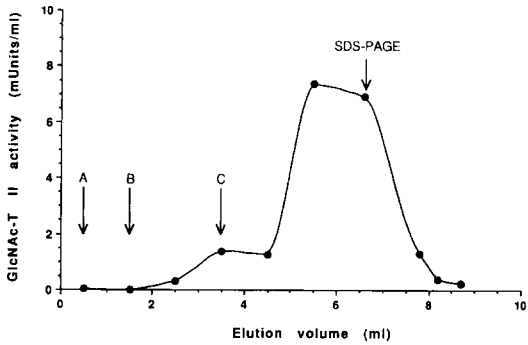


Fig. 5. Chromatography of recombinant human GlcNAc-T II on UDP-GlcNAc-5-propylamine (**8**)-Sepharose. GlcNAc-T II was applied to the column in load buffer as described under Experimental. The column was washed with the load buffer (arrow A), followed by load buffer containing 0.1 M sodium chloride (arrow B). Most of the activity was eluted with load buffer containing 0.1 M sodium chloride but without imidazole (arrow C). Fractions were monitored for GlcNAc-T II activity and SDS-PAGE analysis was performed as indicated by the arrow.

propylamine (**8**) and Sepharose was found to be much more stable and showed affinity properties similar to the corresponding UDP column (Fig. 5). GlcNAc-T II is retarded on the column in the presence of 0.1 M sodium chloride. About 75% of the applied activity is recovered in the main peak (Fig. 5). SDS-PAGE was performed on the eluate (fraction indicated by the arrow in Fig. 5) and a major band with an apparent molecular mass of 53 kDa was detected (Fig. 4) (the theoretical value for the recombinant enzyme is 55.3 kDa). The specific activity was calculated to be at least 10 μmol/min/mg, based on an estimate of protein content by comparison with standard proteins (Fig. 4). No attempt was made to optimize the purification procedure in this study. The inclusion of imidazole in the wash buffers seemed to increase binding. Decreased binding is observed for GlcNAc-T II when the manganese concentration in the wash buffers was increased from 10 to 25 mM $MnCl_2$. Enzyme activity has been reported to decrease at manganese concentrations above the optimum of ca. 10 mM [12].

In conclusion, both GlcNAc-T I and II bind to the UDP-GlcNAc-5-propylamine (**8**)-Sepharose column. GlcNAc-T II interacts more weakly than GlcNAc-T I. This behavior could be due to unfavourable interactions of GlcNAc-T II with the Sepharose matrix upon binding to the ligand; however, retardation was about the same when the ligand was coupled to a C-9 hydrophobic spacer of ECH-Sepharose (Pharmacia) (results not shown). Similarly, it has been reported that GlcNAc-T II is not strongly retarded on a UDP-GlcNAc-5-Hg-thiopropyl-Sepharose column with ligand concentrations in the same range (6.5 μmol/mL); enzyme, however, did not elute with 0.1 M sodium chloride at a much higher ligand concentration of ca. 20 μmol/mL [8]. It is important to note that high ligand concentrations of 20 μmol UDP or UDP-GlcNAc derivatives/mL gel correspond to a total anionic charge of 40–60 μmol/mL, which is almost half of the ionic capacity of a CM-C-50 Sephadex anion-exchange column (100–130 μmol/mL according to the manufacturer); GlcNAc-T I and II bind to this

anion-exchange column. High ligand concentrations may therefore have an adverse effect on protein purification by increasing non-specific ionic interactions.

The main advantage of UDP-GlcNAc-5-propylamine-Sepharose over the corresponding UDP-GlcNAc-5-Hg-thiopropyl-Sepharose is the stability of the ligand and conjugate, which makes the column reusable and allows the use of low ligand concentrations in order to minimize non-specific ionic interactions. The elution profiles shown in Figs 3 and 5 were reproduced twice for GlcNAc-T I and three times for GlcNAc-T II using the same column with only 8 μmol ligand/mL gel.

3. Experimental

General methods.—TLC was performed on silica gel F_{254} (Merck) with detection by UV absorption. Sephadex G-15 (Pharmacia) was used for gel filtration, AG 2-X8 (200–400 mesh, Bio-Rad Laboratories) for anion-exchange chromatography, silica gel 60 (70–230 mesh, Merck) for silica gel chromatography and Sep-Pak C-18 cartridges (Waters) for reversed-phase chromatography. ^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR spectra at 125.8 MHz, using a Varian Unit-Plus 500 NMR spectrometer. Assignments of ^{1}H and ^{13}C resonances were based on 2D experiments (^{1}H/^{1}H-COSY, ^{1}H/^{13}C-HMQC) and polarization transfer experiments [attached proton test (APT)]. Electrospray mass spectrometry was performed in the positive- and negative-ion modes on a SCIEX API III mass spectrometer (Perkin–Elmer); samples were applied by flow injection with water as eluent at 20 μL/min. UV spectra were recorded on a Hitachi U2000 spectrophotometer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [28]; aliquots were mixed with Laemmli buffer containing mercaptoethanol and boiled for 3 min. The gels were fixed and stained by the silver staining method [29], using Silver Stain Plus (Bio-Rad Laboratories) according to the manufacturer's instructions. UDP-[^{3}H]GlcNAc was purchased from American Radiolabeled Chemicals. GlcNAc-T I acceptor M_3-octyl was obtained by chemical synthesis [30]. GlcNAc-T II acceptor GnM_3-octyl was obtained by enzymatic conversion of M_3-octyl with UDP-GlcNAc and recombinant GlcNAc-T I [31].

Uridine 5-[(3-amino)-1-propenyl]-5'-(trihydrogen diphosphate) (**3**) *and uridine 5-[(3-amino)-1-*D,L*-propanol-yl]-5'-(trihydrogen diphosphate)* (**5**).—Compounds **3** and **5** were synthesized following the procedure by Meikle et al. [20] with modifications. UDP was converted into **1** according to Dale et al. [13], with modifications. UDP (1 g, 2.1 mmol) and mercuric acetate (1.9 g, 6.0 mmol) were dissolved in sodium acetate buffer (80 mL, 0.5 M, pH 7.0) and the solution was heated to 50°C. After 2 h, only traces of UDP were detected by TLC (2:1 acetonitrile–water). The reaction mixture was cooled to 4°C and ice-cold absolute ethanol (320 mL) was added. After 30 min the precipitate was collected by centrifugation and the supernatant, which contained excess mercuric acetate, was discarded. The pellet containing **1** was dissolved in water (10 mL), precipitation with ethanol (40 mL) and centrifugation were repeated, and the precipitate was dried in vacuo. Compound **1** was used without further purification in the next reaction step.

Allylamine (1.4 mL, 19 mmol) was mixed with ice-cold sodium acetate buffer (40 mL, 0.5 M, pH 4.0) and the pH was immediately adjusted to pH 5.0 with dil HCl (1 M). Compound **1**, as obtained above, was dissolved in this solution and potassium tetrachloropalladate(II) (1 g, 3.1 mmol) dissolved in water (2 mL) was added. The reaction mixture was stirred rapidly at room temperature overnight to complete the formation of a black metal precipitate. The mixture was concentrated in vacuo, the residue was dissolved in ice-cold absolute ethanol (100 mL), and chloroform (20 mL) was added to precipitate nucleotide products. After 30 min the precipitate was collected by centrifugation and was dissolved in water (5 mL). Precipitation (with absolute ethanol, 30 mL) and centrifugation were repeated. The pellet was dissolved in water (10 mL) and the black metal deposit was removed by filtration over a 0.45 μm membrane.

The filtrate was loaded on an AG 2X-8 anion-exchange column (220 mL, chloride form). The column was washed with water (100 mL), and **3** and **5** eluted together with 100 mM sodium chloride. Fractions containing a mixture of **3** and **5** were pooled and concentrated to saturation of sodium chloride. Water (one volume) and absolute ethanol (ten volumes) were added. The mixture of **3** and **5** crystallized overnight at 4°C. Crystals were dissolved in water (10 mL) and residual salt was removed by gel filtration on a G-15 Sephadex column (300 mL) with water as eluent to give 462 mg of the sodium chloride salts of **3** and **5** (39% yield relative to UDP) which are present in a molar ratio of 5:3 (from integration of the uridyl H-6 signals in the ^{1}H NMR spectrum). Compound **5** is a 1:1 mixture of the two diastereomeric propyl alcohols.

Electrospray MS, negative ion: *m/z* 458.2 ($M-H^-$ for **3**, +0.16, 100%), 476.2 ($M-H^-$ for **5**, +0.15, 73%); ^{1}H NMR (D_2O, HDO = 4.8): **3**: δ 8.21 (s, 1 H, H-6), 6.58 (d, 1 H, *J* 16.0 Hz, C*H*=CHCH$_2$), 6.50 (dt, 1 H, *J* 16.0 Hz, *J* 6.2 Hz, C*H*=C*H*CH$_2$), 6.02–5.99 (m, 1 H, H-1′), 4.50–4.38 (m, 2 H, H-3′, H-2′), 4.38–4.23 (m, 3 H, H-4′, H-5a′, H-5b′), 3.74 (d, 2 H, *J* 6.2 Hz, C*H*$_2$NH$_2$); **5**: δ 8.08 (s, 2 H, H-6′), 6.02–5.99 (m, 2 H, H-1′), 4.80 (m_c, 2 H, C*H*OHCH$_2$CH$_2$), 4.50–4.38 (m, 4 H, H-3′, H-2′), 4.38–4.23 (m, 6 H, H-4′, H-5a′, H-5b′), 3.23–3.10 (m, 4 H, C*H*$_2$NH$_2$), 2.25 and 2.16 (2 × m_c, 4 H, C*H*$_2$CH$_2$NH$_2$) (both diastereomers gave identical ^{1}H NMR spectra, with the exception of the signals for C*H*$_2$CH$_2$NH$_2$, which were resolved in two multiplets); ^{13}C NMR (D_2O, $CH_3COO^-Na^+$ = 25.85): **3**: δ 166.74, 153.36 (2 × C=O), 140.48 (C-6), 128.31 (*C*H=CHCH$_2$), 124.82 (CH=*C*HCH$_2$), 113.78 (C-5), 91.47 (C-1′), 85.69 (C-4′), 76.89 (C-2′), 71.45 (C-3′), 66.68 (C-5′), 43.65 (CH$_2$NH$_2$); **5**: δ 166.42, 153.88 (4 × C=O), 140.37, 140.22 (2 × C-6), 118.72, 118.65 (2 × C-5), 91.37, 91.28 (2 × C-1′), 85.63 (2 × C-4′), 76.70, 76.52 (2 × C-2′), 71.64, 71.57 (2 × C-3′), 67.54, 67.42 (2 × *C*HOHCH$_2$CH$_2$), 66.68 (2 × C-5′), 39.43 (2 × CH$_2$NH$_2$), 34.61, 34.35 (2 × *C*H$_2$CH$_2$NH$_2$).

Uridine 5-[(3-amino)-propyl]-5′-(trihydrogen diphosphate) (**7**).—Compounds **3** and **5** (462 mg, in a molar ratio of 5:3, as obtained above) were dissolved in a mixture of methanol, water, and acetic acid (20 mL, 5:5:1), and hydrogenated at normal pressure on Pd/C (100 mg, 10%) at room temperature. After 3 h, the reaction mixture was filtered through a 0.45 μm membrane. The filtrate was concentrated in vacuo to 5 mL, the pH was adjusted with sodium hydrogencarbonate buffer (0.5 M, pH 9) to pH 6.5, and the product was desalted over a Sephadex G-15 column (300 mL) with water as eluent to give 450 mg of **7** (94%), sodium acetate salt, λ_{max} 267 nm, $\epsilon \approx 10\,000$; $C_{12}H_{21}N_3O_{12}P_2$;

electrospray MS, negative ion: m/z 460 ($M - H^-$, 100%); ^1H NMR (D_2O, HDO = 4.8): δ 7.91 (s, 1 H, H-6), 6.03 (d, 1 H, $J_{1',2'}$ 4.5 Hz, H-1'), 4.47 (dd, 1 H, $J_{2',3'}$ 5.0 Hz, $J_{3',4'}$ 4.5 Hz, H-3'), 4.40 (dd, 1 H, H-2'), 4.32-4.23 (m, 3 H, H-4', H-5a', H-5b'), 3.03 (t, 2 H, J 7.0 Hz, CH_2NH_2), 2.49 (t, 2 H, J 7.5 Hz, $CH_2CH_2CH_2NH_2$), 1.93 (m_c, 2 H, $CH_2CH_2NH_2$).

Uridine 5-[3-(4-benzoylbenzamido)-propyl]-5'-(trihydrogen diphosphate) (**9**).—Compound **7** (10 mg, 17 μmol) was dissolved in phosphate buffer (0.5 mL, 20 mM, pH 4.5) and 4-benzoylbenzoic acid *N*-succinimidyl ester [22] (8 mg, 25 μmol), dissolved in oxolane (300 μL), was added. The reaction was initiated by addition of sodium hydrogencarbonate buffer (0.3 mL, 0.5 M, pH 9.3) and was rapidly stirred at room temperature. After 6 h amine was no longer detectable (TLC, 3:2:1 methanol–chloroform–water) and the pH was adjusted to ca. 6.5 by addition of phosphate buffer (0.5 mL, 0.5 M, pH 4.5). The mixture was concentrated to dryness in vacuo, dissolved in water (1 mL) and precipitated with ice-cold absolute ethanol (6 mL). The precipitate was collected by centrifugation, dissolved in water (2 mL) and purified over a C-18 column (Sep-Pak cartridge) with water as eluent to give 10 mg of the sodium salt of **9** (80%); $C_{26}H_{29}N_3O_{14}P_2$; electrospray MS, negative ion: m/z 668.2 ($M - H^-$, +0.1, 8%); ^1H NMR (D_2O, HDO = 4.8): δ 7.90–7.73 (m, 8 H, Bz, H-6), 7.63–7.58 (m, 2 H, Bz), 5.90 (d, 1 H, $J_{1',2'}$ 4.5 Hz, H-1'), 4.43 (m_c, 1 H, H-3'), 4.38 (m_c, 1 H, H-2'), 4.25 (m_c, 3 H, H-4', H-5a', H-5b'), 3.47 (m, 2 H, CH_2NH), 2.52 (m, 2 H, $CH_2CH_2CH_2NH$), 1.94 (m, 2 H, CH_2CH_2NH).

*Uridine 5-[(3-amino)-1-propenyl]-5'-(trihydrogen diphosphate) mono-α-*D*-N-acetylglucosaminopyranosyl ester* (**4**) *and uridine 5-[(3-amino)-1-*D,L*-propanol-yl]-5'-(trihydrogen diphosphate) mono-α-*D*-N-acetylglucosaminopyranosyl ester* (**6**).—UDP-GlcNAc (1 g, 1.54 mmol) was converted with mercuric acetate (1.4 g, 4.4 mmol) according to Bendiak and Schachter [8], employing the modifications described under the synthesis of **1**. TLC (3:2:1 methanol–chloroform–water) showed complete conversion of UDP-GlcNAc after 6 h at 50°C. Minor by-products were detected. Compound **2**, as obtained above, was alkylated with allylamine (1.1 mL, 15 mmol) and potassium tetrachloropalladate(II) (0.71 g, 2.2 mmol) as described for **3** and **5**, but the allylamine buffer was adjusted to pH 6.0. The reaction was allowed to proceed overnight at room temperature and completion was monitored by TLC (3:2:1 methanol–chloroform–water). The pH was adjusted to ca. 7 with 1 M sodium hydroxide and the mixture was concentrated in vacuo. The residue was diluted with absolute ethanol (100 mL), upon which the nucleotide sugar products precipitated. The precipitate was collected by centrifugation, dissolved in water, and filtered through a 0.45 μm membrane. The filtrate was concentrated in vacuo to ca. 10 mL and desalted over a Sephadex G-15 column (300 mL) with water as eluent. Anion-exchange chromatography on an AG 2-X8 column (220 mL), with 0.1 M sodium chloride as eluent, and desalting with a Sephadex G-15 column, gave 416 mg of a mixture of the sodium chloride salts of **4** and **6** (36% yield from UDP-GlcNAc), which are present in a molar ratio of 5:2 (from integration of the uridyl H-6 signals in the ^1H NMR spectrum). Compound **6** was a 1:1 mixture of the two diastereomeric propyl alcohols.

Electrospray MS, negative ion: m/z 660.8 ($M - H^-$ for **4**, −0.3, 100%), 679.2 ($M - H^-$ for **6**, +0.08, 21%); ^1H NMR (D_2O, HDO = 4.8): **4**: δ 8.14 (s, 1 H, H-6),

6.61 (d, 1 H, *J* 16.0 Hz, C*H*=CHCH$_2$), 6.52 (dt, 1 H, *J* 16.0 Hz, *J* 6.5 Hz, CH=C*H*CH$_2$), 6.03–6.01 (m, 1 H, H-1′), 5.55 (dd, 1 H, $J_{1'',2''}$ 3.5 Hz, $J_{1'',P\beta}$ 7.0 Hz, H-1″), 4.43–4.39 (m, 2 H, H-3′, H-2′), 4.36–4.28 (m, 2 H, H-5a′, H-5b′), 4.28–4.22 (m, 1 H, H-4′), 4.03–3.98 (m, 1 H, H-2″), 3.95 (ddd, 1 H, $J_{5'',6a''}$ 2.3 Hz, $J_{5'',6b''}$ 4.25 Hz, $J_{4'',5''}$ 10 Hz, H-5″), 3.91–3.80 (m, 3 H, H-6a″, H-6b″, H-3″), 3.75 (d, 2 H, *J* 6.5 Hz, C*H*$_2$NH$_2$), 3.57 (dd, 1 H, $J_{3'',4''}$ 10 Hz, H-4″), 2.10 (s, 3 H, CH$_3$CO); **6**: δ 8.01, 8.00 (2 × s, 2 H, H-6), 4.80 (m$_c$, 2 H, C*H*OHCH$_2$CH$_2$), 3.24–3.12 (m, 4 H, C*H*$_2$NH$_2$), 2.19 and 2.10 (2 × m$_c$, 4 H, C*H*$_2$CH$_2$NH$_2$) (data for the ribose and GlcNAc moieties of both diastereomers of **6** are identical with those for **4**).

*Uridine 5-[(3-amino)-propyl]-5′-(trihydrogen diphosphate) mono-α-*D-N-*acetylglucosaminopyranosyl ester* (**8**).—**4** and **6** (28 mg, in a molar ratio of 5:2, as obtained above) were dissolved in a mixture of methanol, water, and acetic acid (3 mL, 5:5:1), and were hydrogenated over Pd/C (10 mg, 10%) as described for **7** (3 h, TLC: 3:2:1 methanol–chloroform–water, R_f(**6**) < R_f(**8**), to give 27 mg of the sodium acetate salt of **8** (94%), λ_{max} 267 nm, $\epsilon \approx 10\,000$; $C_{20}H_{34}N_4O_{17}P_2$; electrospray MS, negative ion: *m/z* 662.8 (M − H$^-$, −0.33, 100%); positive ion: *m/z* 665.0 (MH$^+$, −0.15, 100%), 687.0 (MNa$^+$, −0.13, 29%), 462 [(M − HexNAc + 2H)$^+$, 33%]; ^{1}H NMR (D$_2$O, HDO = 4.8): δ 7.87 (s, 1 H, H-6), 6.03 (d, 1 H, $J_{1',2'}$ 4.2 Hz, H-1′), 5.54 (dd, 1 H, $J_{1'',2''}$ 3.3 Hz, $J_{1'',P\beta}$ 7.26 Hz, H-1″), 4.43–4.38 (m, 2 H, H-2′, H-3′), 4.34–4.26 (m, 2 H, H-5a′, H-5b′), 4.26–4.20 (m, 1 H, H-4′), 4.01 (m$_c$, 1 H, H-2″), 3.95 (ddd, 1 H, $J_{5'',6a''}$ 2.2 Hz, $J_{5'',6b''}$ 4.2 Hz, $J_{4'',5''}$ 10.1 Hz, H-5″), 3.89 (dd, 1 H, $J_{6a'',6b''}$ 12.5 Hz, H-6a″), 3.83 (dd, 1 H, $J_{3'',4''}$ 10.6 Hz, H-3″), 3.83 (dd, 1 H, H-6b″), 3.57 (dd, 1 H, H-4″), 3.05 (t, 2 H, *J* 7.3 Hz, C*H*$_2$NH$_2$), 2.50 (m$_c$, 2 H, C*H*$_2$CH$_2$CH$_2$NH$_2$), 2.10 (s, 3 H, CH$_3$CO), 1.92 (m$_c$, 2 H, C*H*$_2$CH$_2$NH$_2$).

*Uridine 5-[3-(4-benzoylbenzamido)-propyl]-5′-(trihydrogen diphosphate) mono-α-*D-N-*acetylglucosaminopyranosyl ester* (**10**).—Compound **8** (17 mg, 22 μmol) was converted with 4-benzoylbenzoic acid *N*-succinimidyl ester [22] (10 mg, 31 μmol) as described for **9**. The reaction mixture was neutralized and transferred with water (50 mL) and ethyl acetate (50 mL) into a separating funnel, the aqueous phase was concentrated to dryness in vacuo, and the residue was dissolved in water (1 mL) and purified over a C-18 Sep-Pak column with water as eluent to give 15 mg of the sodium salt of **10** (80%); $C_{34}H_{42}N_4O_{19}P_2$; electrospray MS, negative ion: *m/z* 871.2 (M − H$^-$, +0.02, 70%), 435.2 [(M − 2H)$^{2-}$, +0.13, 100%); positive ion: *m/z* 895.2 (MNa$^+$, +0.02, 81%), 873.2 (MH$^+$, 100%), 670 [(M − HexNAc + 2H)$^+$, 35%], 590 [(M − HexNAcP + 2H)$^+$, 7%], 510 [(M − HexNAcPP + H)$^+$, 29%], 378 [(M − HexNAcPPPent + 2H)$^+$, 15%]; ^{1}H NMR (D$_2$O, HDO = 4.8): δ 7.91–7.86 (m, 2H, B z), 7.84–7.80 (m, 2 H, Bz), 7.79–7.75 (m, 3 H, Bz, H-6), 7.70 (m$_c$, 1 H, Bz), 7.56 (m$_c$, 2 H, Bz), 5.90 (d, 1 H, $J_{1',2'}$ 3.0 Hz, H-1′), 5.45 (m$_c$, 1 H, H-1″), 4.34 (m$_c$, 2 H, H-2′, H-3′), 4.19 (m$_c$, 3 H, H-4′, H-5a′, H-5b′), 3.96–3.69 (m, 5 H, H-2″, H-3″, H-5″, H-6a″, H-6b″), 3.49–3.40 (m, 3 H, H-4″, C*H*$_2$NH), 2.47 (t, 2 H, *J* 7.1 Hz, C*H*$_2$CH$_2$CH$_2$NH), 2.01 (s, 3 H, CH$_3$CO), 1.87 (m$_c$, 2 H, C*H*$_2$CH$_2$NH).

Recombinant GlcNAc-T I and II.—The catalytic domain of rabbit GlcNAc-T I used in this work was a recombinant enzyme expressed in the baculovirus/Sf9 insect cell system as described by Sarkar [3] and was purified to near homogeneity with a specific activity of about 10 μmol/min/mg as previously described [3]. This preparation

showed a major band at 42 kDa on SDS-PAGE, derived by proteolytic cleavage of a 50 kDa protein which is present as a minor component (lane 4, Fig. 4). The recombinant human GlcNAc-T II used in this work was also expressed in the baculovirus/Sf9 insect cell system [27]. The preparation was a fusion protein (theoretical molecular mass 55.3 kDa) of the entire human GlcNAc-T II protein (cytoplasmic tail, trans-membrane domain, stem region, and catalytic domain), a $(His)_6$ sequence upstream of the amino-terminal end and an enterokinase cleavage site. The enzyme was partially purified by affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) to a specific activity of 0.1–0.2 μmol/min/mg [27] and contained many bands (lane 7, Fig. 4).

Kinetic experiments and enzyme assays.—GlcNAc-T I and II were assayed radiochemically using Pasteur pipette columns of AG1-X8 (Cl^- form, 100–200 mesh) to separate radiolabelled product from unreacted radiolabelled sugar-nucleotide donor UDP-GlcNAc [32]. The incubation mixture used for kinetic studies with GlcNAc-T I contained, in a total volume of 0.025 mL: 0.02 munit (2.1 ng) of enzyme (1 unit = 1 μmol/min), 50 nmol of acceptor octyl *O*-(α-D-mannopyranosyl)-(1 → 3)-*O*-[α-D-mannopyranosyl-(1 → 6)]-β-D-mannopyranoside (M_3-octyl) 2.5–20 nmol of UDP-[^{3}H]GlcNAc (9670 dpm/nmol), 2–4 nmol of inhibitor (when present and as indicated), 30 mM MES [2-(*N*-morpholino)ethanesulfonic acid monohydrate], pH 6.1, 20 mM $MnCl_2$, bovine serum albumin (25 μg), Triton X-100 (0.1%, v/v), and glycerol (4%, v/v). The standard assay conditions for GlcNAc-T I were as above except for the use of 20 nmol M_3-octyl and 20 nmol UDP-[^{3}H]GlcNAc (9670 dpm/nmol). The incubation mixture used for kinetic studies with GlcNAc-T II contained, in a total volume of 0.025 mL: 0.023 munit (115 ng) of enzyme (1 unit = 1 μmol/min), 10 nmol of acceptor octyl -(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1/2)-*O*-(α-D-mannopyranosyl)-(1 → 3)-*O*-(α-D-mannopyranosyl-(1 → 6)]-β-D-mannopyranoside (GnM_3-octyl), 5–40 nmol of UDP-[^{3}H]GlcNAc (9670 dpm/nmol), 2–5 nmol of inhibitor (when present and as indicated), 25 mM MES, pH 6.8, 13 mM $MnCl_2$, bovine serum albumin (25 μg), Triton X-100 (0.1%, v/v) and glycerol (4%, v/v). The standard assay conditions for GlcNAc-T II were as above except for the use of 6 nmol of GnM_3-octyl and 20 nmol of UDP-[^{3}H]GlcNAc (9670 dpm/nmol). Samples were incubated for 60 min at 37°C and the reaction was stopped by the addition of 0.4 mL of ice-cold water.

Coupling of the amines **7** *and* **8** *to Sepharose.*—Compounds **7** and **8** were coupled to cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's instructions, using 20 μmol ligand/mL gel in 0.5 M $NaHCO_3$ buffer at pH 9.3. Yields were between 6.5 and 13 μmol ligand/ml gel, based on the recovery of unreacted ligand after coupling.

Affinity chromatography of GlcNAc-T I.—A column of UDP-GlcNAc-5-propylamine-Sepharose (3 mL, 8 μmol **8**/mL gel) was equilibrated with buffer A (25 mM MES, pH 6.5, 20% glycerol, 0.1% Triton X-100, 10 mM $MnCl_2$, 0.02% NaN_3). GlcNAc-T I (63 munit, ca. 6 μg of protein, in 1 mL of buffer A) was applied at a flow-rate of ca. 6 mL/h. The column was washed with buffer A containing 0.1 M sodium chloride (7 mL), followed by buffer A containing 0.5 M sodium chloride (5 mL). GlcNAc-T I (45 munit) was eluted with buffer A containing 0.1 M sodium chloride and 5 mM UDP-GlcNAc (10 mL).

Affinity chromatography of GlcNAc-T II.—A column of UDP-GlcNAc-5-propylamine-Sepharose (3 mL, 8 μmol **8**/mL gel) was equilibrated with buffer B (25 mM MES, pH 7.0, 25 mM imidazole, 20% glycerol, 0.1% Triton X-100, 10 mM $MnCl_2$, 0.02% NaN_3). GlcNAc-T II (24 munit, 266 μg of protein, in 0.5 mL of buffer B as above but without $MnCl_2$) was applied at a flow-rate of ca. 4 mL/h. The column was washed with buffer B (1 mL), followed by buffer B containing 0.1 M sodium chloride (2 mL) and buffer B containing 0.1 M sodium chloride but without imidazole (7 mL). Most of the enzyme activity (18 munit, ca. 3 μg of protein) was recovered in a 2 mL fraction during the last wash step.

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