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Note

The use of a synthetic dideoxygenated pentasaccharide as a specific acceptor for *N*-acetylglucosaminyltransferase-III

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Mammalian glycosyltransferases assemble oligosaccharides by transferring single sugar residues from a sugar-nucleotide (the donor) to a growing carbohydrate chain (the acceptor)[1,2]. The availability of an acceptor substrate is therefore a minimum requirement for assaying the activity of these enzymes.

Both isolated and synthetic oligosaccharide substrates have been used in glycosyltransferase assays, which normally quantitate the transfer of ^{3}H or ^{14}C labelled sugars to produce radioactive products[1]. When such assays are performed with crude extracts of biological tissues many glycosyltransferases are usually present, and several of these can frequently act on the same oligosaccharide acceptor[3]. More than one product can therefore be formed and quantitation of individual glycosyltransferase activities requires tedious product separation[4]. In such instances, synthetic carbohydrate chemistry can play an especially important role, since monospecific acceptor oligosaccharides can be prepared where the OH groups glycosylated by interfering enzymes can be either removed[5,6] or masked[7] (for example, by *O*-methylation). In all cases where such "unnatural" modified acceptors are used in radioactive assays, the potential for artifactual reactions exist. The isolation and structural characterization of the product formed from unnatural acceptors is therefore essential for the validation of such assays[8].

The synthetic pentasaccharide octyl glycoside **1** is an example of a structure which is a potential substrate for three competing *N*-acetylglucosaminyltransferases, termed[3] GlcNAc-transferases III, IV and V. GlcNAc-transferase III (GlcNAcT-III)[9], an enzyme whose activity is elevated in several liver disorders[10], would convert **1** into the bisected hexasaccharide **2** where, in the

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natural acceptor glycopeptides, the octyl group would be replaced by a chitobiosyl-asparagine residue. In an effort to make this acceptor monospecific for GlcNAcT-III, we chemically synthesized its dideoxy analog **3**, in which the OH groups that would become glycosylated by GlcNAcT-IV and -V are absent[11]. Pentasaccharide **3** was found to incorporate radiolabelled GlcNAc on incubation of crude rat kidney extracts (a known source of GlcNAcT-III) with the radiolabelled donor UDP[^{3}H]GlcNAc (see Fig. 1). We report herein the isolation and structural characterization of the product hexasaccharide **4**, thereby confirming the validity of using **3** as an acceptor for the assay of GlcNAcT-III.

EXPERIMENTAL

Methods and materials.—Pentasaccharide **3** was available from previous work[11]. Rat kidneys were from Pel-Freeze (Rogers, AR). Uridine 5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl diphosphate) (UDP-GlcNAc), adenosine-5′-monophosphate (AMP), $MnCl_2$, *N*-acetyl-α-D-glucosamine (GlcNAc), 4-morpholine ethanesulfonic acid (MES), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and Triton X-100 were from Sigma (St. Louis, MO). UDP[^{3}H]GlcNAc was from Du Pont

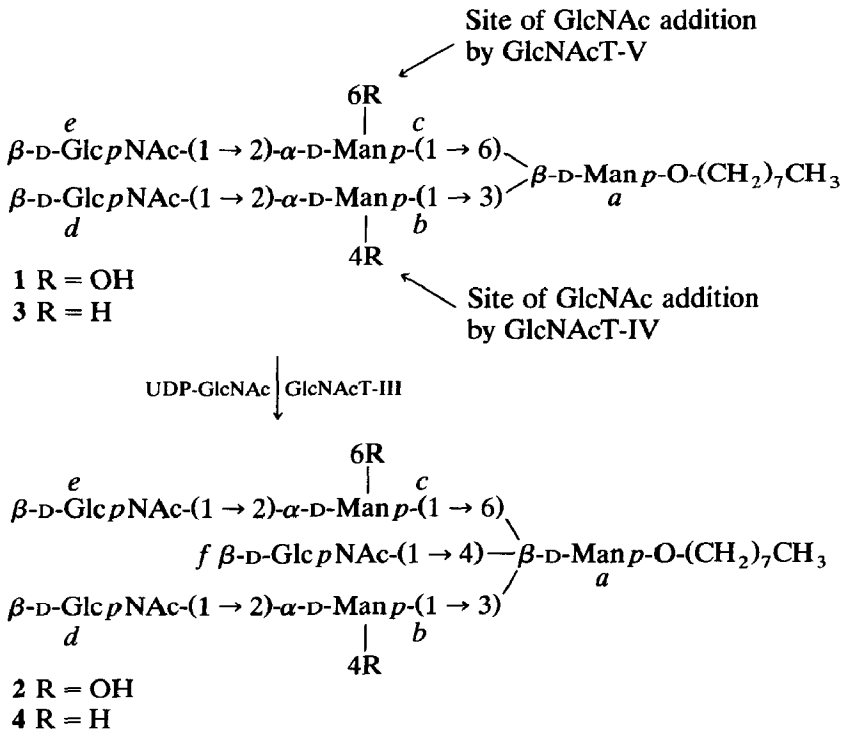

Fig. 1. Schematic reaction diagram for GlcNAcT-III. The letters *a*–*f* are labels for each sugar residue.

(Mississauga, ON). Ecolite scintillation cocktail was from ICN Biomedicals (Irvine, CA). C_{18} Sep Pak cartridges were from Waters (Mississauga, ON). All other chemicals were ACS reagent grade or, for chromatography, HPLC grade. The HPLC equipment used was a Waters (Mississauga, ON) model 590 pump, a model U6K injector, and a model 481 UV detector. The HPLC column was a Whatman (Clifton, NJ) Partisil-5-PAC, 0.47×11 cm. All HPLC and NMR samples were filtered with Millex-HV 0.45-μm filters from Millipore (Mississauga, ON). ^{1}H NMR were recorded with a Varian UNITY 500 MHz spectrometer. Solutions were in 99.996% D_2O and chemical shifts were referenced to external acetone at 2.225 ppm. Mass spectra were recorded using fast-atom bombardment as previously described[11]. The following buffers were used. Buffer *A*: 125 mM MES, 125 mM GlcNAc, 1% Triton X-100, 5 mM AMP, and 10 mM $MnCl_2$ (pH 5.9). Buffer *B*: 250 mM MES, 395 mM GlcNAc, 2% Triton X-100, 10.6 mM AMP, and 26.4 mM $MnCl_2$ (pH 5.9).

Enzyme isolation.—GlcNAcT-III was prepared following an established method[12]. Briefly, 50 rat kidneys (36 g) were homogenized in a Waring blender with 200 mL of 10 mM Tris-HCl (pH 7.4) and 0.15 M NaCl. This homogenate was centrifuged at 900*g* for 12 min at 4°C. The resulting supernatant (18.1 mg/mL protein) was used for the following procedures.

Kinetic enzyme assays.—UDP-GlcNAc (1.2 μmol), 0.0029–0.153 μmol pentasaccharide **3**, and 1.44×10^6 dpm UDP[^{3}H]GlcNAc were added as aqueous solutions to 0.5-mL microfuge tubes, and lyophilized to dryness. Each tube had 20 μL (36.2 μg protein) of enzyme extract and 40 μL of buffer *A* added just prior to incubation for 3 h at 37°C. After incubation, the mixture was diluted by the addition of 400 μL of ice-cold water followed by adsorption of the radiolabelled product onto C_{18} cartridges. The cartridges were washed with 50 mL of water and the product hexasaccharide was eluted with 10 mL MeOH. The amount of radioactivity was determined by scintillation counting on a Beckman LS1801 instrument with automatic quench correction. The kinetic parameters were determined using a computer program employing Wilkinson's method with 8 different concentrations of acceptor[13]. The kinetic data are shown in Fig. 2.

Preparative enzyme incubation.—To a 1.5-mL microcentrifuge tube, 14.5 mg of UDP-GlcNAc and 1.0 mg (1.0 μmol) of pentasaccharide **3** were added as dry powders. Enzyme extract (0.5 mL) and buffer *B* (0.5 mL) were added to this tube, and incubated at ambient temperature. After 3 days a further 9.8 mg of UDP-GlcNAc and 0.3 mL of enzyme supernatant were added. After 5 more days the reaction was terminated by the addition of 50 mL of water and loading onto a C_{18} cartridge. The cartridge was washed with 100 mL of water and the product was eluted with 15 mL of MeOH. This eluant was concentrated to dryness by rotary evaporation at 40°C with a water aspirator vacuum, and further purified by HPLC with a PAC column. The solvent was 4:1 CH_3CN–H_2O and the flow rate was 1.0 mL/min. The sample was injected in water and the column effluent was monitored at 202 nm.

RESULTS AND DISCUSSION

When pentasaccharide **3** was used as the glycosyltransferase acceptor, and crude rat kidney extracts as the enzyme source, incorporation of labelled GlcNAc from UDP-GlcNAc proceeded according to Michaelis–Menten kinetics (see Fig. 2). This result was expected for a single enzyme utilizing **3** as the acceptor. The K_m for **3** was determined to be 0.65 ± 0.06 mM and V_{max} was 1492 ± 55 pmol/min/mg under the conditions used (data not shown). This K_m is somewhat higher than the previously published value[12] of 0.19 mM for a natural acceptor that is similar in structure to **1**, but contains the chitobiosyl unit. The enzyme activity was completely abolished by addition of EDTA (10 mM), a characteristic[10] of GlcNAcT-III.

A preparative incubation of **3** (1 μmol) with the enzyme extract was purified by reverse-phase C_{18} HPLC. Only 2 oligosaccharides attached to a hydrophobic octyl group were observed and subsequently isolated: starting pentasaccharide **3** (0.37 μmol) with a retention time of 6.56 min, and the new product (designated **4**, 0.59 μmol) with a retention time of 9.25 min. These yields are approximate since they are based on HPLC peak integrations (A_{202}) with the assumption that all the GlcNAc residues have equal extinction coefficients. Essentially all of acceptor **3** was thus accounted for.

The new product **4** was shown to be a hexasaccharide by ^{1}H NMR spectroscopy which indicated the addition of a β-linked GlcNAc residue to **3**. This was confirmed by FAB mass spectrometry, where **3** produced a molecular ion (M + Na, 78%) at m/z 990, while product **4** showed this ion (M + Na, 19%) at 1193. The formation of an octyl dideoxyhexasaccharide was thereby demonstrated.

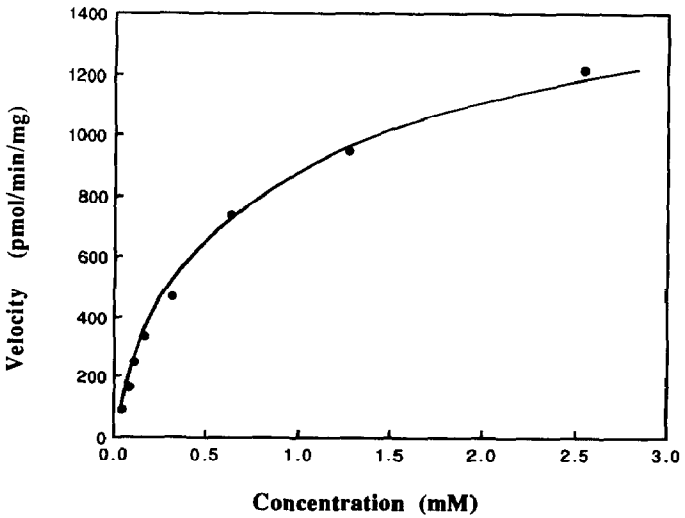

Fig. 2. Michaelis–Menten plot for GlcNAcT-III acting on dideoxy acceptor **3**.

TABLE I

500-MHz ^{1}H NMR chemical shifts (ppm) for compounds **3** and **4** [a]

Compound	*a*	*b*	*c*	*d*	*e*	*f*
3 H-1	4.692	5.175	4.887	4.589	4.607	
				$J_{1,2}$ 8.2 [b]	$J_{1,2}$ 9.0	
H-2	4.14	4.05	4.17	3.72	3.702	
NHAc				2.069	2.069	
4 H-1	4.638	5.122	4.982	4.612	4.607	4.473
				$J_{1,2}$ 8.4	$J_{1,2}$ 8.4	$J_{1,2}$ 8.3
H-2	4.21	4.08	4.19	3.71	3.71	3.69
H-4		1.55*ax*				
		1.69*eq*				
NHAc				2.079	2.079	2.088

[a] The letters above each row refer to the sugar labelling in Fig. 1. [b] The coupling constants are reported as observed splittings in Hz.

Hexasaccharide **4** was assigned the bisected structure, shown, on the basis of its ^{1}H NMR 1D and 2D COSY spectra (Table I and Fig. 3). The spectrum of the reporter proton region of **4** shows the presence of a doublet (J 8.3 Hz) at δ 4.473, which is a shift uniquely observed in the bisecting βGlcNAc(1 → 4) residue in complex natural oligosaccharides[14]. All other features of the NMR spectrum (Table I), including characteristic H-2 chemical shifts, are also in accord with those of the natural structures[15] except, of course, signals for protons near the reducing

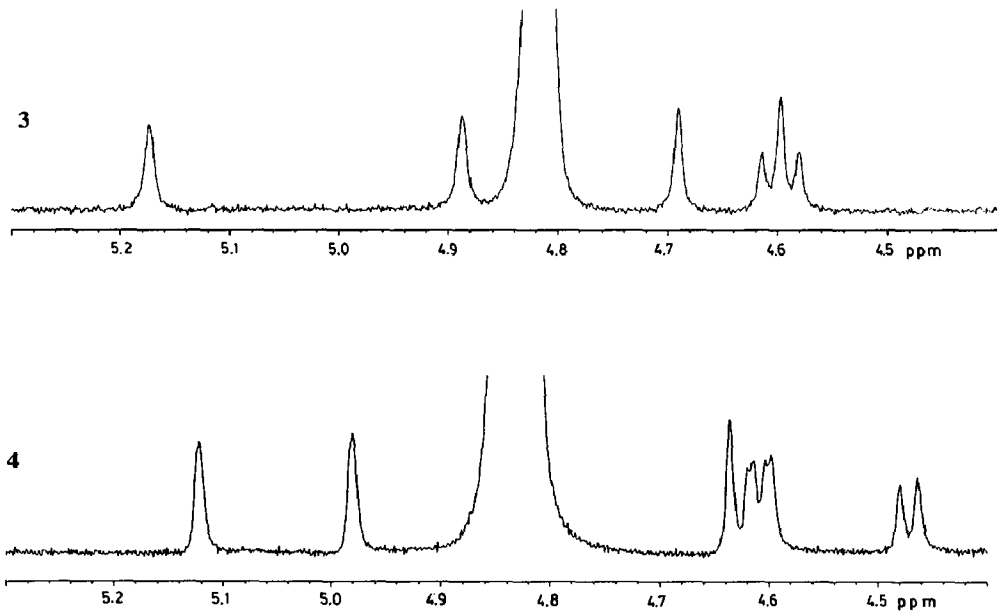

Fig. 3. Partial 500 MHz ^{1}H NMR spectra of dideoxy acceptor **3** and dideoxy product **4**. Only the anomeric region is shown.

end βMan residue where compounds **3** and **4** are attached to the unnatural octyl aglycon. The conclusion reached from this work is that synthetic dideoxy acceptor pentasaccharide **3** is a kinetically competent substrate for GlcNAcT-III. Compound **3** should therefore be useful for quantitating this enzyme activity in crude biological specimens using the convenient "Sep Pak" assay[16].

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