Unexpected Enzymatic Fucosylation of the Hindered Tertiary Alcohol of 3-*C*-Methyl-*N*-Acetyllactosamine Produces a Novel Analogue of the LeX-Trisaccharide

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Received September 25, 1997

Mammalian oligosaccharides are biosynthesized through the action of glycosyltransferases that sequentially transfer single pyranosyl residues from nucleotide mono- or diphosphate sugars to growing carbohydrate chains.[1] The increasing availability of recombinant glycosyltransferases has resulted in a corresponding increase in the use of these enzymes to achieve efficient combined chemical−enzymatic syntheses of such oligosaccharides.[2] Numerous examples have also appeared where glycosyltransferases have been shown to either transfer an *unnatural* sugar to the natural substrate oligosaccharide or transfer a natural sugar to an *unnatural* oligosaccharide substrate, thus increasing the scope of the enzymatic step to include the preparation of oligosaccharide analogues.[3]

Most oligosaccharide analogues that have been synthesized to probe the molecular specificity of carbohydrate-protein recognition have the hydroxyl groups on the pyranose rings either derivatized or replaced with other functional groups.[4] Very few carbon-branched sugar residues have been reported[5] and even fewer where the branching occurs at a carbon bearing a glycosylated OH-group.[6,7] This is undoubtedly because the chemical glycosylation of complex hindered tertiary alcohols is notoriously difficult.[8] The scope of analogues that have been prepared using glycosyltransferases also does not yet include sugar residues that are branched at the carbon bearing the hydroxyl group undergoing glycosylation. Since such carbon-branched sugar units do not exist in mammalian oligosaccharides, it could be expected that the available glycosyltransferases might not be able to catalyze their formation. Oligosaccharides containing such branched sugar units would be of interest for probing which face of a given sugar is making contact with a protein binding site. The resulting glycosidic linkage to a tertiary alcohol would additionally be expected to be conformationally much less flexible than that to the natural secondary OH group.

We report here the chemical synthesis of the *N*-acetyllactosamine analogue **3** bearing a methyl branch at C-3 of the GlcNAc residue. This compound was found to be a kinetically competent acceptor for a fucosyltransferase that transfers an α-Fuc residue to the branched alcohol, yielding the trisaccharide analogue **4** of the well-known blood group LeX trisaccharide **2**. Remarkably, **4** was also found to be an excellent substrate for a fucosidase known to act on the natural LeX structure **2**.

The glycosyl acceptor **6** was glycosylated by reaction of the glycosyl donor **5**,[9] promoted by AgOTf, yielding the expected *β*-linked disaccharide **7** (81%) (Scheme 2). The *O*-allyl group was then removed using $PdCl_2$, providing **8** in quantitative yield. Oxidation of **8** was achieved using DMSO−Ac_2O. Treatment of the resulting ketone with methyllithium gave compound **9**, with an axial *C*-methyl group.[11] *O*-Deacetylation with NaOMe in MeOH followed by hydrogenolysis using $Pd(OH)_2$ provided **3.** The configuration of C-3 was established through NMR TROESY studies which show the significant NOE between 3-*C*-methyl protons and H-1 of the GlcNAc residue.

Disaccharide **1** is a known acceptor for the α(1→3/4)-fucosyltransferase[12] that can be readily isolated from human milk.[13] The K_m value for **1** was found to be 0.4 mM using an established radioactive "Sep-Pak assay",[14] and the relative velocity of fucosyl transfer was arbitrarily set to 100. The *C*-methyl-branched acceptor **3** was also found to be an acceptor in the same radioactive assay, with 20-fold elevated K_m (to 8.0 mM) but a 70% increase in V_{rel} (synthesis) (Scheme 1).

It was very surprising that the enzyme tolerated the introduction of a *C*-methyl group directly at the site of transfer, implying that there was substantial flexibility in the active site at the transition state of the reaction. To be certain that the reaction proceeded normally, i.e. that OH-3 was indeed the alcohol that became fucosylated, a preparative reaction was performed[15] to yield the *C*-methyl-branched trisaccharide **4**, whose structure was confirmed by ^{1}H NMR and MS analyses.[16] In particular, H-5 of the newly

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(10) The ketone product was not isolated but reacted directly in the next step.

(11) Selected ^{1}H NMR data for **3** (500 MHz, D_2O): *δ* 4.55 (d, 1H, *J* = 8.8 Hz, H-1), 4.47 (d, 1H, *J* = 7.8 Hz, H-1′), 3.92 (d, 1H, *J* = 3.2 Hz, H-4′), 1.22−1.34 (m, 13H, $(CH_2)_5CH_3$ and C-3-CH_2). HR-ESMS *m/e* calcd for $(M + H^+)$ $C_{23}H_{44}NO_{11}$: 510.2914. Found: 510.2915.

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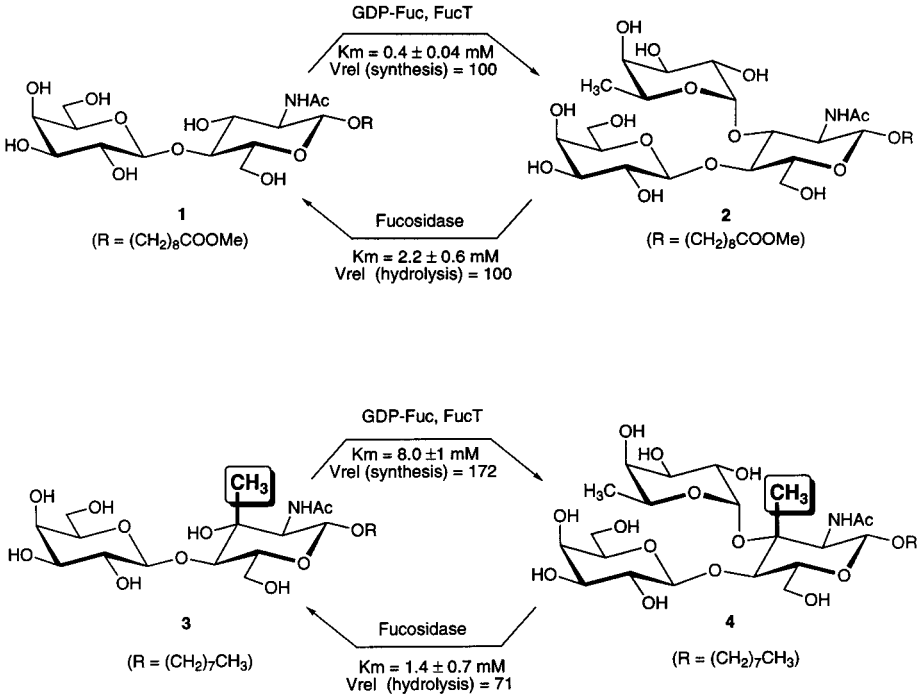
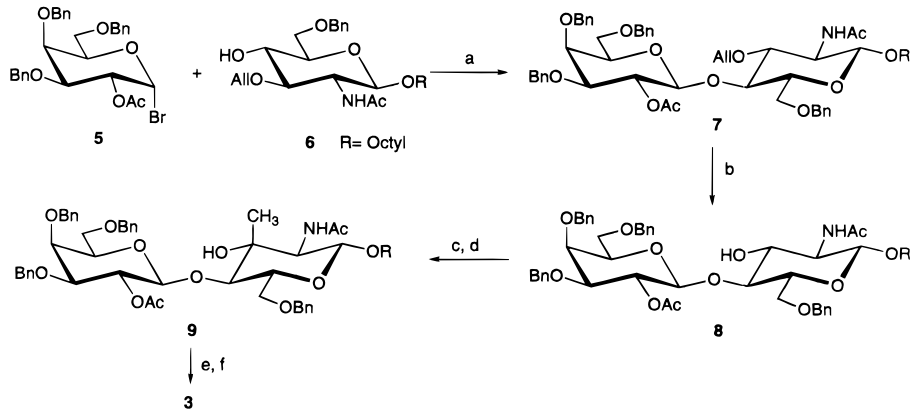
(15) The reaction mixture contained **3** (1.0 mg, 2.0 μmol), GDP-Fuc (1.7 mg, 2.9 μmol), 30 mU of milk FucT (in 300 μL of 25 mM sodium cacodylate buffer, pH 6.5, containing 5 mM $MnCl_2$ and 25% glycerol), 30 μL of concentrated buffer (200 mM Hepes, pH 7.0, containing 200 mM $MnCl_2$, and 2% BSA), and 3 μL of calf intestine alkaline phosphatase (1 U/μL). The reaction was incubated at 37 °C with rotation for 2 days, then at room temperature for 4 days. Additional GDP-Fuc was added daily (total 1.6 mg). The reaction was stopped by filtering through a 0.22-μm Millex GV filter unit, and product **4** was isolated by loading the mixture onto two sequential Sep-Pak C18 reverse phase cartridges. The cartridges were washed with water to remove enzyme and unreacted nucleotide donor, with 30% aqueous methanol to remove guanosine, and then with 50% aqueous MeOH to elute **4** (1.0 mg, 75% yield).

(16) Selected ^{1}H NMR data for **4** (600 MHz, D_2O): *δ* 5.29 (d, 1H, *J* = 4.0 Hz, H-1″), 4.68 (bq, 1H, H-5″, *J* = 6.8 Hz), 4.49 (d, 1H, *J* = 8.4 Hz, H-1), 4.48 (d, 1H, *J* = 7.7 Hz, H-1′). HR-ESMS *m/e* calcd for $(M + H^+)$ $C_{29}H_{54}NO_{15}$: 656.3493. Found: 656.3498.

Scheme 1



Scheme 2[a]



[a] Reagents and Conditions: (a) **3** (1.5 equiv), AgOTf (2 equiv), 4 Å molecular sieves, CH_2Cl_2, −30 to 0 °C, 3 h, 81%; (b) $PdCl_2$ (0.5 equiv), MeOH, room temperature (rt), 2 h, quantitative; (c) DMSO, Ac_2O, rt, 4 h; (d) MeLi (1.5 equiv), THF, −78 °C, 2 h, 20% (two steps); (e) NaOMe, MeOH, rt, 27 h, 92%; (f) H_2, 20% $Pd(OH)_2$/C, MeOH, 20 h, 85%.

introduced α-Fuc residue was strongly downfield-shifted (to 4.68 ppm), which is diagnostic of 3-*O*-fucosyl-*N*-acetyllactosamine sequences such as **2**, where it is found near 4.8 ppm.[17]

Having access to the *C*-methyl-branched trisaccharide **4**, we also examined if the fucosidase from almond meal that is known[18] to cleave the Fuc residue in the LeX structure **2** would tolerate the introduction of a methyl group so close to the glycosidic oxygen that must be protonated by the enzyme as glycoside hydrolysis is initiated. Remarkably, the introduction of the methyl group was found to have very little effect on the kinetics of the hydrolysis of **4**, resulting in improved recognition of the substrate (with a 40% decrease in Km) and only a modest reduction (30%) in the reaction velocity (Scheme 1).[19]

The surprising finding in this work is that both the fucosyltransferase and the fucosidase enzymes tolerate the introduction of a large methyl substituent on the same carbon that bears the oxygen which must be activated in both reactions. It will be interesting to see if this will be the case also for other glycosyltransferases.

Acknowledgment. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (to O.H.) and the Medical Research Council of Canada (to M.M.P.).

Supporting Information Available: Spectral data for compounds **3**, **4**, and **7**−**9** (3 pages). See any current masthead page for ordering and Web access instructions.

JA973361W

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(19) Compounds **2** or **4** (0.1−4 mM) were incubated at 37 °C with 6.3 μU almond meal fucosidase (Sigma, E.C. 3.2.1.111) in 25 μL of 50 mM sodium citrate buffer, pH 5.0, for 24 h. Fucose released was quantitated by removing 10-μL aliquots, transferring them to microtiter wells containing 13 mU of