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βGal(1→S-4)βGlcNAc–OR: A GALACTOSIDASE-STABLE SUBSTRATE FOR α(1→3)FUCOSYLTRANSFERASE

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Abstract: 1′-4 Thio-*N*-acetyllactosamine was chemically synthesized as a galactosidase-stable substrate for α(1→3)fucosyltransferase. The product of enzymatic fucose addition was confirmed to be the thio-Le X analog.

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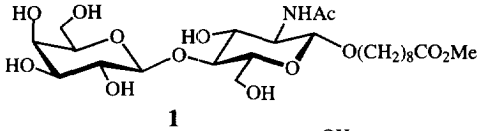
N-Acetyllactosamine is one of the most frequently occurring constituents of glycosphingolipids and glycoproteins in which the carbohydrate moieties often represent epitopes for cellular recognition, signal transduction, and adhesion phenomena.[1] For instance, *N*-Acetyllactosamine (LacNAc) can serve as a recognition site in bacterial adhesion[2] and as a tumor-associated antigen.[3] LacNAc also serves as a substrate for fucosyltransferases, sialyltransferases, and *N*-acetylglucosaminyltransferases involved in the biosynthesis of complex oligosaccharides.

We have previously reported[4] that fluorescently labeled LacNAc derivatives are useful for assaying the biosynthetic activities of glycosyltransferases in crude cell extracts where product formation can be quantitated by capillary electrophoresis with laser-induced fluorescence detection. One of the difficulties in such experiments, however, was that products of degradation initiated by β-galactosidase seriously depleted the LacNAc substrate. We therefore considered two possible analogs (**2** and **3**) of LacNAc (**1**) that might overcome this limitation. Disaccharide **2**, where the ring oxygen of the β-Gal residue was replaced with a sulfur, was available from previous work[5] where it was found to be resistant to β-galactosidase. However, enzymatic assays revealed **2** to be neither an acceptor nor an inhibitor for an α(1→3/4) fucosyltransferase partially purified from human milk. We therefore turned to the synthesis and enzymatic evaluation of the thio-disaccharide **3** where the oxygen in the glycosidic linkage is replaced by sulfur. Such 1-thio-glycosides are known to be resistant to glycosidases.[6]



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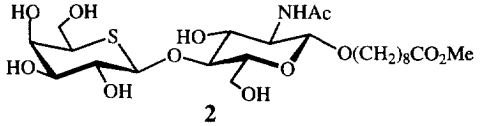

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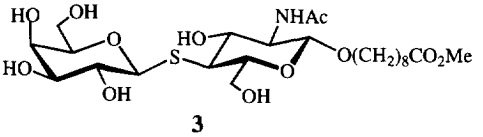

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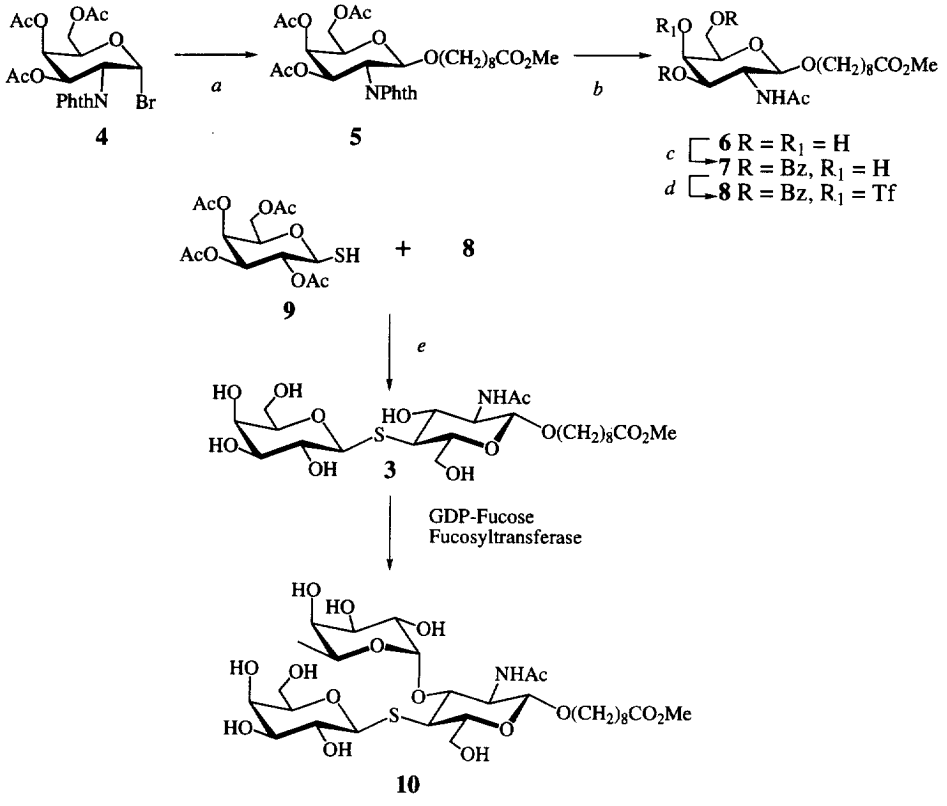
3,4,6-Tri-*O*-acetyl-2-deoxy-2-phthalimido-α-D-galactopyranosyl bromide (**4**) was coupled with 8-methoxycarbonyloctanol in dichloromethane in the presence of AgOTf, affording the β-linked glycoside **5** in 71% yield. *O*-Deacetylation of **5** with NaOMe/MeOH, followed by treatment with hydrazine acetate in ethanol and subsequent *N*-acetylation, yielded 8-methoxycarbonyl-2-acetamido-2-deoxy-β-D-galactopyranoside (**6**) in 58% yield (three steps). Selective benzoylation of **6** with benzoyl chloride (2.2 equiv at -40 °C), gave 3,6-dibenzoate derivative **7** in 63% yield. Reaction with triflic anhydride in pyridine converted **7** into the 4-*O* triflate **8**, which was then coupled with 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose (**9**) in DMF containing NaH at -20 °C. The resulting sulfur-linked disaccharide was deacetylated with NaOMe/MeOH, providing 1′-4 thio-*N*-acetyllactosamine (**3**) in 28% isolated yield (three steps). The ^{1}H NMR signals at 4.43 ppm (d, 1H, J = 9.6 Hz), 4.39 ppm (d, 1H, J = 8.4 Hz) and ^{13}C NMR signals at 102.35 ppm, 86.26 ppm confirmed the structure.



Scheme 1 *a*: 1, $HO(CH_2)_8CO_2Me$, AgOTf, 4 Å MS, 71%. *b*: 1, NaOMe/MeOH; 2, NH_2NH_2-HOAc, EtOH, 70 °C; 3, MeOH, Ac_2O, 58% (1-3, three steps). *c*: Benzoyl chloride (2.2 equiv), -40 °C, 63%. *d*: Tf_2O, pyridine, -10 °C. *e*: **1**, **9** (1.5 equiv), DMF, NaH; 2, NaOMe/MeOH; 38% (three steps).

The sulfur-linked disaccharide **3** was evaluated as a potential acceptor for a partially-purified human milk α(1→3/4) fucosyltransferase that has been extensively characterized and used in synthesis.[7] By using an

established radioactive "Sep-Pak assay",[8] disaccharide **3** was found to be a kinetically competent acceptor displaying a K_m value of 230 μM and V_{max} (20 pmol/min for the enzyme preparation used) was nearly the same (~90%) as that for the parent LacNAc disaccharide **1**. Under the same conditions, **2** was inactive.

To confirm that the expected product was formed, a preparative incubation was performed using 1.2 mg of **3**.[9] The produced trisaccharide **10** showed ^{1}H NMR signals at 5.03 ppm (d, 1H, J = 3.8 Hz), 4.47 ppm (d, 1H, J = 8.4 Hz), 4.46 ppm (d, 1H, J = 8.4 Hz) and 4.62 ppm (q, 1H) confirming the 1′-4-thio-Le X structure.[10]

Finally, the resistance of **3** to β-galactosidase was experimentally verified. No hydrolysis of thiodisaccharide **3** was evident after incubation with 590 mU of β-galactosidase from E.Coli for 30 min under conditions previously reported.[5] Galactose release was monitored using a kit from Boehringer-Mannheim containing 88 mU of galactose dehydrogenase and NAD.[5] Under these conditions, the parent LacNAc **1** (510 μM) was completely hydrolyzed.

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9. The reaction mixture contained **3** (1.2 mg), GDP-Fuc (1.7 mg), 30 mU of milk fucosyltransferase in 400 μL of 25 mM sodium cacodylate buffer, pH 6.5, containing 25 mM $MnCl_2$, 0.2% BSA and 25% glycerol

was incubated at 37 °C for 3 days. Additional GDP-Fuc (1.0 mg) was added after 24 h and again after 48 h. The reaction was stopped by the addition of 8 mL of water and the sample was isolated by loading the mixture onto two sequential C-18 Sep-Pak cartridges (Waters). The cartridges were washed with water to remove enzyme and unreacted nucleotide donor and the product was eluted with methanol to give trisaccharide **10**.

10. Spectral data for selected new compounds: **3**. ^{1}H NMR (360 MHz, CD_3OD): δ 4.43 (d, 1H, J = 9.6 Hz, H′-1), 4.39 (d, 1H, J = 8.0 Hz, H-1), 3.64 (s, 3H, OCH_3), 2.83 (m, 1H, H-2), 1.96 (s, 3H, $NHCOCH_3$); ^{13}C NMR (300 MHz, CD_3OD): δ 102.35 (C-1), 86.26 (C-1′), 81.01, 78.20, 75.95, 73.59, 70.67, 70.48 (C-3, C-5, C-2′, C-3′, C-4′ and C-5′), 70.07 (OCH_2), 63.31, 62.84 (C-6 and C-6′), 58.57 (C-2), 51.95 (OCH_3), 23.02 ($NHCOCH_3$); HRMS for $C_{24}H_{43}NO_{12}SNa$ $[M+Na]^+$ 592.2404, found: 592.2408. **6**. ^{1}H NMR (360 MHz, CD_3OD): δ 4.35 (d, 1H, J = 8.4 Hz, H-1), 3.64 (s, 3H, OCH_3), 1.95 (s, 3H, $NHCOCH_3$). **7**. ^{1}H NMR (360 MHz, CD_3OD): δ 5.86 (d, 1H, J = 8.9 Hz, N*H*), 5.44 (dd, 1H, H-3), 4.76 (d, 1H, J = 8.3 Hz, H-1), 3.66 (s, 3H, OCH_3), 3.52 (m, 1H, H-2), 1.89 (s, 3H, $NHCOCH_3$); ^{13}C NMR (300 MHz, CD_3OD): δ 101.05 (C-1), 74.24, 72.16, 66.74 (C-3, C-4 and C-5), 69.48 (OCH_2), 63.35 (C-6), 50.58 (C-2), 51.26 (OCH_3), 22.85 ($NHCOCH_3$). **8**. ^{1}H NMR (360 MHz, CD_3OD): δ 6.05 (dd, 1H, H-3), 5.48 (d, 1H, J = 2.9 Hz, H-4), 5.17 (d, 1H, J = 8.3 Hz, H-1), 3.65 (s, 3H, OCH_3), 1.88 (s, 3H, $NHCOCH_3$). **10**. ^{1}H NMR (360 MHz, CD_3OD): δ 5.03 (d, 1H, J = 3.8 Hz, H-1″), 4.47 (d, 1H, J = 8.4 Hz, H-1), 4.46 (d, 1H, J = 8.4 Hz, H-1′), 4.62 (q, 1H, H-5″), 3.64 (s, 3H, OCH_3), 2.30 (t, 2H, CH_2CO), 1.94 (s, 3H, $NHCOCH_3$); FABMS: *m/z* 738 $[M+Na]^+$.