High-Efficiency Synthesis of Sialyloligosaccharides and Sialoglycopeptides

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Abstract: Sialyllactosamine structures were synthesized in high yield from carbohydrates and glycopeptides containing terminal GlcNAc with a two-step enzymatic glycosylation in a "one-pot" reaction. A key improvement is the use of alkaline phosphatase to destroy nucleotide phosphate inhibitors generated in the glycosyltransferase reaction.

The biological importance of sialosides has been well documented,[1] and the need for model compounds has posed synthetic challenges to carbohydrate chemists. Despite recent progress in the chemical synthesis of sialosides,[2] the enzymatic approach[3] is potentially more efficient in the rapid formation of naturally occurring sialyloligosaccharides.[4] Increasing availability of glycosyltransferases through cloning techniques[5] will have a strong influence on oligosaccharide synthesis in the foreseeable future. A multigram synthesis for CMP-*N*-acetylneuraminic acid[6] has been published.

Enzymatic glycosylation is still limited because enzymes and sugar nucleotides are expensive and should be used efficiently. This has been recognized by Wong et al.[7] who used a sophisticated recycling system that generated UDP-galactose (**2**) from the product UDP, which is also a potent inhibitor of the reaction.

Here we report the high-yield synthesis of sialyllactosamine structures **6a-d** as diagrammed in Figure 1.

Experimental Section

Materials. UDP-glucose, bovine serum albumin, UDPglucose 4'-epimerase (EC 5.1.3.2), and bovine galactosyltransferase (EC 2.4.1.22) were purchased from Sigma. Calf intestinal alkaline phosphatase (EC 3.1.3.1) was obtained from Boehringer Mannheim. The Galβ1,4GlcNAc α-2,6-sialyltransferase (EC 2.4.99.1) was purified as described previously.[8] CMP-*N*-acetylneuraminic acid was prepared according to reference.[6] ^{1}H NMR spectra were recorded in deuterium oxide on a Bruker AM 360/Wb. Thin-layer chromatography was performed on silica gel plates (60-F254; E. Merck, Darmstadt) and visualized by spraying with 1 N H_2SO_4 in ethanol containing 0.1% orcinol.

(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranulosonic acid)-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxyglucopyranose (6a). A 5-mg portion (22.7 μmol) of *N*-acetylglucosamine (**3a**) was dissolved in 565 μL (50 mM) of sodium cacodylate (pH 7.4) containing 0.5 mg of bovine serum albumin, 1.1 μmol of $MnCl_2$, 3.4 μmol of NaN_3, 28.3 μmol of UDP-glucose, 200 milliunits of GlcNAc β-1,4-galactosyltransferase (EC 2.4.1.22), 1 unit of UDPglucose 4'-epimerase (EC 5.1.3.2), and 4 units of calf intestinal alkaline phosphatase (EC 3.1.3.1). The reaction mixture was incubated at 37 °C, and the pH was maintained at 7.4 by periodic addition of 0.25 N NaOH. After 48 h, 1.7 mL of H_2O, 30 μmol of CMP-NeuAc, 1.4 mg of bovine serum albumin, 10 μmol of NaN_3, 100 milliunits of β-galactoside α-2,6-sialyltransferase (EC 2.4.99.1), and 6 units of calf intestinal alkaline phosphatase were added. Incubation was continued for 2 days at 37 °C with the pH at 7.4. Sialoside **6a** was isolated by gel chromatography on a Sephadex G-25 superfine column (2.3 × 32 cm) by eluting with 0.1 M NH_4HCO_3. The fractions (3 mL) containing **6a** (TLC in 1 M NH_4OAc/isopropyl alcohol, 1/2.4) were pooled and lyophilized. ^{1}H NMR data were in accord with those reported:[5] yield 11.4 mg (16.9 μmol; 74.4% calculated from starting material **3a**).

Azido(5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranulosonic acid)-(2,6)-β-D-galactopyranosyl-(1,4)-2-deoxy-β-D-glucopyranose (6b). A 5.6-mg portion (22.7 μmol) of β-azido *N*-acetylglucosamine (**3b**)[14c] was reacted according to the procedure for the synthesis of **6a**: yield 11.4 mg (16.3 μmol; 72% calculated from starting material **3b**); ^{1}H NMR δ 4.45 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, H-1'), 2.67 (dd, 1 H, $J_{3eq'',4''}$ = 4.7 Hz, $J_{3eq'',3ax''}$ = 12.2 Hz, H-3eq''), 2.09, 2.03 (2 s, 6 H, NAc, NAc''), 1.72 (t, 1 H, $J_{3ax'',4''} = J_{3eq'',3ax''}$ = 12.2 Hz, H-3ax'').

N^4-[(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranulosonic acid)-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-N^2-[(allyloxy)carbonyl-L-phenylalanyl]-L-asparaginyl-L-seryl-L-threonyl-L-isoleucine (6c). A 19.7 mg portion (22.7 μmol) of N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-N^2-[[(allyloxy)carbonyl]-L-phenylalanyl]-L-asparaginyl-L-seryl-L-threonyl-L-isoleucine (**3c**)[14c] and 4 mg of bovine serum albumin were reacted according to the procedure for the synthesis of **6a**: yield 24.7 mg (18.7 μmol, 82.4% calculated from starting material **3c**); ^{1}H NMR δ 7.33 (m, 5 H, aromat), 5.9 (m, 1 H, =CH- allyl), 2.68 (m, 2 H, β-CH_2b Asn, H-3eq''), 2.05, 2.03 (2 s, 6 H, NAc, NAc''), 1.73 (t, 1 H, $J_{3ax'',4''} = J_{3eq'',3ax''}$ = 12.1 Hz, H-3ax''), 1.24 (d, 3 H, J = 6.1 Hz, CH_3 Thr), 0.91 (m, 6 H, CH_3 Ile).

N^4-[(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranulosonic acid)-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-N^2-(glycylglycyl)-L-asparaginylglycylglycine (6d). A 12.8 mg portion (22.7 μmol) of N^4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-N^2-(glycylglycyl)-L-asparaginylglycylglycine (**3d**) was reacted according to the procedure for the synthesis of **6a**: yield 19.8 mg (19.5 μmol; 86% calculated from starting material **3d**); ^{1}H NMR δ 5.1 (d, 1 H, $J_{1,2}$ = 9.4 Hz, H-1), 4.44 (d, 1 H, $J_{1',2'}$ = 8.3 Hz, H-1'), 2.85 (m, 2 H, β-CH_2 Asn), 2.66 (dd, 1 H, $J_{3eq'',4''}$ = 4.6 Hz, $J_{3eq'',3ax''}$ = 12.2 Hz, H-3eq''), 2.03, 2.01 (2 s, 6 H, NAc, NAc''), 1.7 (t, 1 H, $J_{3ax'',4''} = J_{3eq'',3ax''}$ = 12.2 Hz, H-3eq'').

Results

It has been shown previously[9] that *N*-acetylglucosamine (**3a**) can be converted to sialyllactosamine (**6a**) in a single step by subsequent enzymatic transfer of galactose and sialic acid. The reaction efficiency could be increased by two improvements: First, the use of alkaline phosphatase to destroy nucleotide phosphate inhibitors that are released during the glycosyltransferase reaction and, second, the use of conditions that minimize the hydrolysis of nucleotide sugars.

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(1) (a) Kawasaki, T.; Ashwell, G. *J. Biol. Chem.* **1977**, *252*, 6536-6543. (b) Schauer, R. *Adv. Carbohydr. Chem. Biochem.* **1980**, *40*, 131-234. (c) Paulson, J. C. In *The Receptors*; Conn, M., Ed.; Academic Press: New York, 1985; Vol. 2, p 131-219.

(2) (a) Murase, T.; Ishida, H.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1989**, *188*, 71-80. (b) Ito, Y.; Numata, M.; Sugimoto, M.; Ogawa, T. *J. Am. Chem. Soc.* **1989**, *111*, 8508-8510.

(3) Toone, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 5365-5422.

(4) (a) Sabesan, S.; Paulson, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 2068-2080. (b) Thiem, J.; Treder, W. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1096-1097. (c) De Heij, H. T.; Kloosterman, M.; Koppen, P. L.; van Boom, J. H.; van den Eijnden, D. H. *J. Carbohydr. Chem.* **1988**, *7*, 209-222. (d) Augé, C.; Gautheron, C.; Pora, H. *Carbohydr. Res.* **1989**, *193*, 288-293. (e) Nilsson, K. G. *Carbohydr. Res.* **1989**, *188*, 9-17. (f) Palcic, M. M.; Venot, A. P.; Ratcliffe, R. M.; Hindsgaul, O. *Carbohydr. Res.* **1989**, *190*, 1-11.

(5) Review: Paulson, J. C.; Colley, K. C. *J. Biol. Chem.* **1989**, *264*, 17615-17618.

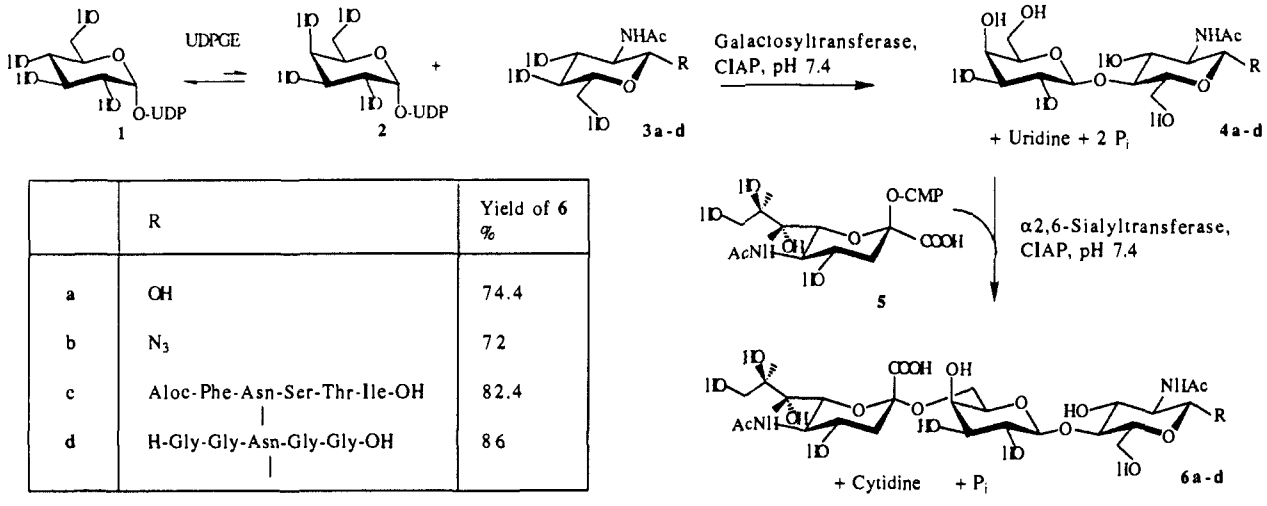
(6) Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *J. Am. Chem. Soc.* **1988**, *110*, 7159-7163.

(7) Wong, C. H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 5416-5418.

(8) Weinstein, J.; de Souza-e-Silva, U.; Paulson, J. C. *J. Biol. Chem.* **1982**, *257*, 13835-13844.

(9) Paulson, J. C.; Rearick, J. I.; Hill, R. J. *J. Biol. Chem.* **1977**, *252*, 2363-2371.

0002-7863/90/1512-9308$02.50/0



	R	Yield of 6 %
a	OH	74.4
b	N_3	72
c	Aloc-Phe-Asn-Ser-Thr-Ile-OH	82.4
d	H-Gly-Gly-Asn-Gly-Gly-OH	86

Figure 1.

Calf Alkaline Phosphatase (CIAP) Improves the Yield of the Galactosyltransferase Reaction

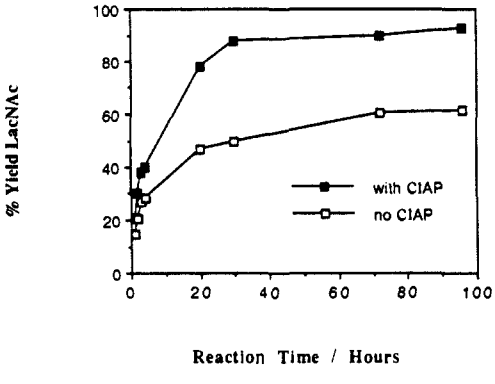


Figure 2.

The UDP-galactose (**2**) required for the galactosyltransferase reaction is generated in situ by UDPgalactose 4′-epimerase (UDPGE), an enzyme that converts inexpensive UDP-glucose (**1**) into UDP-galactose (**2**), while an equilibrium of Glc/Gal = 3.5/1 is maintained. Since UDP-galactose (**2**) is subject to degradation by Mn^{2+} in alkaline media,[10] the concentration of Mn^{2+} was kept low (2 mM) and neutral pH (7.4) was maintained. A 25% excess of nucleotide sugar (50 mM) over the acceptor (40 mM) assured the presence of sufficient amounts of donor substrate even toward the end of the reaction.

To evaluate the inhibitory effect of UDP on the final yields of disacchardide **4a**, the galactosyltransferase reaction was examined with and without the presence of calf intestinal alkaline phosphatase (CIAP, molecular biology grade). As seen in Figure 2, the use of alkaline phosphatase gives a dramatic improvement, leading to a higher reaction velocity over the entire time course and permits near quantitative conversion of the acceptor employed. Since nucleotide phosphates are generally product inhibitors of glycosyltransferases, alkaline phosphatase may be of general use to accelerate enzymatic oligosaccharide synthesis.

Following galactosylation, sialic acid addition could be achieved without intermediate purification. For optimal yields, the reaction mixture was diluted 4-fold and adjusted to pH 7.4 with 0.25 N NaOH. This was necessary since the α-2,6-sialyltransferase is inhibited by salt concentrations higher than 100 mM[11] and there is significant hydrolysis of CMP-NeuAc at pH <7.4.[12] Sialylation was then accomplished by addition of sialyltransferase, CMP-NeuAc, and CIAP, followed by incubation at 37 °C for an additional 48 h. As for galactosylation, the yields for the sialyltransferase reaction were improved by addition of CIAP.

By avoiding the isolation of the intermediate galactoside, the above reaction scheme leads to a rapid and very efficient synthesis of sialosides that are part of the terminal region of N-linked carbohydrate groups of glycoproteins, structures where sialic acid is known to have a key role in bioactivity.[1] Two monosaccharides (**3a,b**) and two synthetic glycopeptides (**3c,d**) were used as acceptors in the "single-pot" buildup of sialyllactosamine compounds **6a–d**, which could be purified easily by gel chromatography on Sephadex G-25. The glycopeptide acceptors **3c,d** were synthesized following two different strategies.[13] Compound **3c** was obtained by liquid-phase synthesis with selective deprotection methods for Aloc-[14] and *tert*-butyl ester groups. According to the work of Lavielle et al.,[15] **3d** was obtained by solid-phase glycopeptide synthesis on a Merrifield resin with Boc-protected amino acids and a final HF cleavage. Glycopeptides with carbohydrate structure like **6c,d** have been isolated from patients with the rare disease aspartylglucosaminuria.[16] Even though the K_m value for the galactosylation of **3d** is unfavorable (18.2 mM) compared with *N*-acetylglucosamine (5 mM[17]), the overall yield of 86% for **6d** is surprisingly high, giving an average glycosylation yield of 92.7% in each step. Enzymatic synthesis of sialyllactosaminylasparagine was recently demonstrated by Augé et al.[4d] by using separate glycosylation steps, which gave a moderate overall yield.

The reaction sequence presented in this report can easily be scaled up to any desired size, limited only by the availability of enzymes and nucleotide sugars. Furthermore, the amount of enzyme needed might be reduced by reusing immobilized enzyme.

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Registry No. **1**, 133-89-1; **2**, 2956-16-3; **3a**, 7512-17-6; **3b**, 29847-23-2; **3c**, 129922-14-1; **3d**, 129922-15-2; **5**, 3063-71-6; **6a**, 78969-47-8; **6b**, 129942-99-0; **6c**, 129943-00-6; **6d**, 129943-01-7; CIAP, 9001-78-9; UDPGE, 9032-89-7; galactosyltransferase, 9030-11-9; α2,6-dialyltransferase, 9075-81-4.

(10) Nunez, H. A.; Barker, R. *Biochemistry* **1976**, *15*, 3843–3847.
(11) Weinstein, J. Personal communication.
(12) Comb, D. J.; Watson, D. R.; Roseman, S. *J. Biol. Chem.* **1966**, *241*, 5637–5642.
(13) Reviews: (a) Garg, H. G.; Jeanloz, R. W. *Adv. Carbohydr. Chem. Biochem.* **1985**, *43*, 135–201. (b) Kunz, H. *Angew. Chem.* **1987**, *99*, 297–311; *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 294–308.
(14) (a) Kunz, H.; Unverzagt, C. *Angew. Chem.* **1984**, *96*, 426–427; *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 436–437. (b) Kunz, H.; Unverzagt, C. *Angew. Chem.* **1988**, *100*, 1763–1765; *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1697–1699. (c) Unverzagt, C. Dissertation, Mainz, 1988.
(15) Lavielle, S.; Ling, N. C.; Guillemin, R. *Carbohydr. Res.* **1981**, *89*, 221–228.
(16) Review: Strecker, G.; Montreuil, J. *Biochimie* **1979**, *61*, 1199–1246 and references cited herein.
(17) Barker, R.; Olsen, K. W.; Shaper, J. H.; Hill, R. L. *J. Biol. Chem.* **1972**, *247*, 7135–7147.