Synthesis of Galactose-Terminated Oligosaccharides by Use of Galactosyltransferase[1]

Joachim Thiem,* Torsten Wiemann

Institut für Organische Chemie, Universität Hamburg, Martin-Luther-King-Platz 6, D-2000 Hamburg 13, Germany

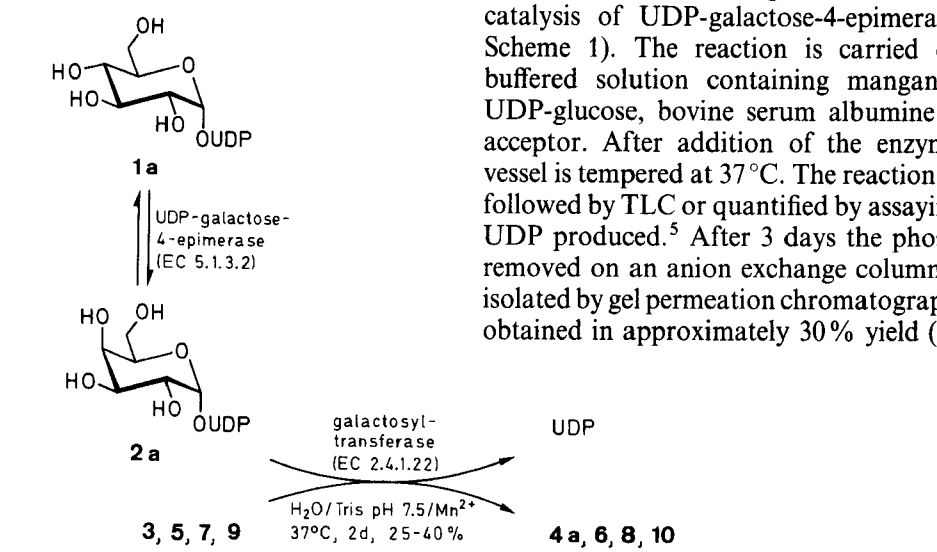
Received 12 August 1991

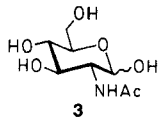
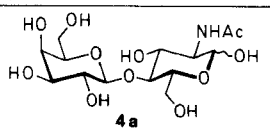
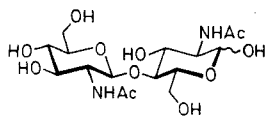
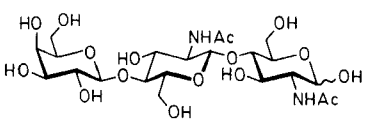
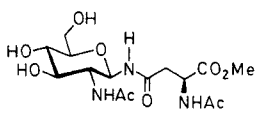
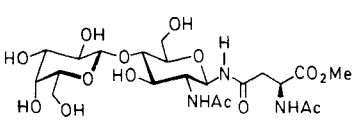
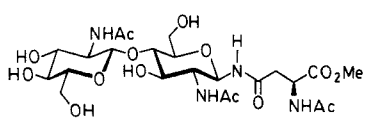
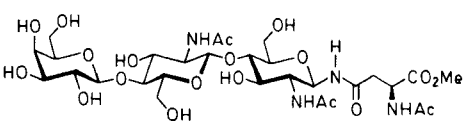
Galactosyltransferase catalyzes the galactosylation of oligosaccharides terminated by glucose and by 2-acetamido-2-deoxy glucopyranose, respectively. Variations concerning the acceptor substrate as well as the donor substrate are described.

Due to their prominent importance in biological processes carbohydrates became the object of intensive research in the last decades. The synthesis of glycosides has been achieved with great success.[2] However, elaborate blocking group chemistry and usually cumbersome work-up procedures are the disadvantages of classical oligosaccharide synthesis. In some cases the employment of enzymes turned out to be a convenient way to overcome these difficulties.[3]

The majority of enzymes used in oligosaccharide synthesis belong to the subclasses of glycohydrolases and glycosyltransferases. Employing the former their transglycosylation potential may be used advantageously, however, this results in a mixture of two or three regioisomers. The latter needs nucleotide activated sugar donors which are transferred to a sugar acceptor giving a single product with well defined regio- and stereochemistry. Among the glycosyltransferases the uridine-5′-diphosphogalactose: D-glucose 4β-galactosyl-transferase (GalT, EC 2.4.1.22) is comparatively easy to obtain and hence most intensively studied.

We have used this enzyme for the synthesis of several oligosaccharides related to partial structures of glycoproteins. The transferase requires uridine-5′-diphosphogalactose (UDP-Gal, **2a**) as a glycosyl donor. Previously, it was shown by Nunez and Barker[4] that this transfer may be carried out on a preparative scale. Since UDP-Gal is very expensive it is advantageous to start with UDP-glucose (**1a**) and generate the former in situ by catalysis of UDP-galactose-4-epimerase (EC 5.1.3.2.; Scheme 1). The reaction is carried out in degassed buffered solution containing manganese(II) chloride, UDP-glucose, bovine serum albumine (BSA), and the acceptor. After addition of the enzymes the reaction vessel is tempered at 37 °C. The reaction turnover may be followed by TLC or quantified by assaying the amount of UDP produced.[5] After 3 days the phosphate esters are removed on an anion exchange column. The product is isolated by gel permeation chromatography and is usually obtained in approximately 30% yield (Table 1).



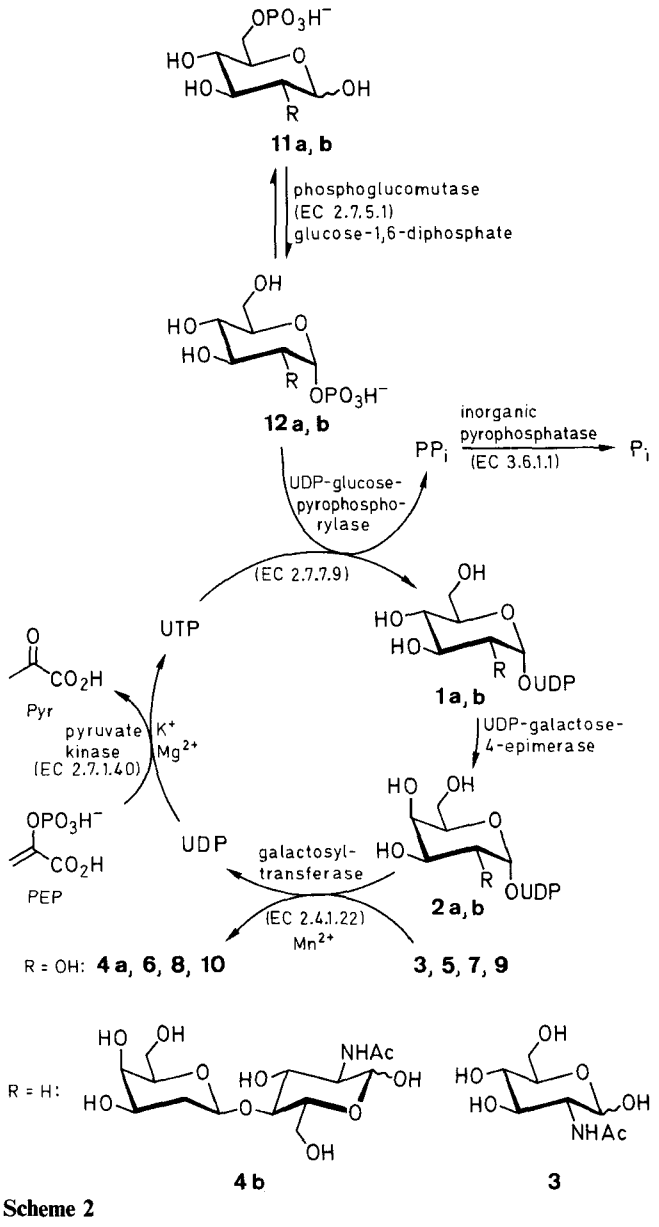
substrates	products
 3	 4a
 5	 6
 7	 8
 9	 10

Scheme 1

Table. Oligosaccharides Prepared

Product	Yield (%)	^{1}H NMR (D_2O) δ, J (Hz) (selected data)
4a	28	5.19 (d, H-1, α-form), 4.71 (d, H-1, β-form), 4.46 (d, H-1'), 3.95 (dd, H-6a), 3.53 (ddd, H-5), 3.65 (ddd, H-5'), 2.03 (s, NAc) $J_{1,2}(\alpha) = 2.2$, $J_{1,2}(\beta) = 7.5$, $J_{4,5} = 7.7$, $J_{5,6a} = 3.8$, $J_{5,6b} = 10.1$, $J_{6a,6b} = 10.1$, $J_{1',2'} = 7.5$, $J_{4',5'} = 1.7$, $J_{5',6a'} = 3.1$, $J_{5',6b'} = 10.2$ Identical with an authentic sample.
6	40	5.20 (d, H-1, α-form), 4.71 (d, H-1, β-form), 4.62 (d, H-1'), 4.48 (d, H-1''), 2.05 (s, NAc), 2.08 (s, NAc) $J_{1,2}(\alpha) = 2.7$, $J_{1,2}(\beta) = 8.1$, $J_{1',2'} = 7.9$, $J_{1'',2''} = 7.8$
8	35	5.06 (d, H-1), 4.49 (d, H-1'), 2.95 (dd, β-Asn), 2.82 (dd, β'-Asn), 2.03, 2.02 (each s, each NAc) $J_{1,2} = 9.4$, $J_{1',2'} = 7.6$, $J_{\alpha,\beta} = 5.6$, $J_{\alpha,\beta'} = 7.6$, $J_{\beta,\beta'} = 17.1$
10	28	5.09 (d, H-1), 4.62 (d, H-1'), 4.47 (d, H-1''), 2.96 (dd, β-Asn), 2.88 (dd, β'-Asn), 2.06, 2.02, 2.01 (each s, each NAc) $J_{1,2} = 9.6$, $J_{1',2'} = 8.1$, $J_{1'',2''} = 7.7$, $J_{\alpha,\beta} = 4.0$, $J_{\alpha,\beta'} = 17.0$
14	40	5.22 (d, H-1, α-form), 4.66 (d, H-1, β-form), 4.45 (d, H-1') $J_{1,2}(\alpha) = 3.7$, $J_{1,2}(\beta) = 7.9$, $J_{1',2'} = 7.5$ Identical with an authentic sample.
16	18	5.23 (d, H-1, α-form), 4.67 (d, H-1, β-form), 4.55 (d, H-1'), 4.46 (d, H-1'') $J_{1,2}(\alpha) = 3.9$, $J_{1,2}(\beta) = 7.9$, $J_{1',2'} = 7.9$, $J_{1'',2''} = 7.5$
4b[a]	40	5.26 (d, H-1, α-form), 2.18 (m, H-2'e), 2.09 (s, NHAc), 1.76 (m, H-2'a), H-1 (β-form) and H-1' are covered by HDO-signal $J_{1,2}(\alpha) = 4.2$.

[a] ^{13}C NMR data of free sugar **4b** and ^{1}H NMR of the heptaacetate see Experimental Section.



Scheme 2

The comparatively minor yields are probably due to product inhibition caused by UDP.[6] This disadvantage, however, is certainly outweighed by the absence of any byproducts.

Thus 2-acetamido-2-deoxy-D-glucopyranose (**3a**) is galactosylated to *O*-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**4a**), a disaccharide unit present in virtually all *N*-glycoproteines. Similarly, *N,N'*-diacetylchitobiose (**5**) is converted to the trisaccharide **6**, respectively.[7] The asparagine derivatives **7** and **9** are synthesized following classical routes according to published procedures.[8] Both compounds serve as glycosyl acceptors in the GalT reaction to give **8** and **10**, respectively.[7] In its fully deprotected form the aspartyl-*N*-acetyllactosaminyl derivative **8** appears in the urine of patients suffering from aspartylglucosaminuria.[9]

One disadvantage connected with preparative enzymatic synthesis is the high cost of most enzymes. In many cases a way to a more economic route consists of immobilizing the enzymes for further applications. Additionally, the stability against temperature, pH-value and ionic strength is generally increased. We have immobilized the epimerase and the transferase von VA-Epoxy[10] as well as on activated CH-Sepharose 4B,[11] the latter being more successful with activity yields in the vicinity of 10%. Using immobilized enzymes the reaction is stopped simply by filtration, rinsing the material and employing it for another reaction cycle.

UDP-glucose is still relatively expensive but it can be generated in situ my mimicking biochemical transformations[12] (Scheme 2, R=OH). Starting from D-glucose 6-phosphate (**11a**) the corresponding glucose α-1-phosphate **12a** is produced by catalysis of phosphoglucomutase (EC 2.7.5.1.). In the presence of uridine-5'-diphosphoglucose pyrophosphorylase (EC 2.7.7.9.) **12a** is condensed with uridine 5'-triphosphate (UTP) to give UDP-glucose. As a byproduct pyrophosphate (PP_i) is formed which, in order to make this reaction irreversible, is hydrolyzed by inorganic pyrophosphatase (EC 3.6.1.1.). The following two steps are identical with those described above. The cycle is closed by phosphorylation of UDP to give UTP. This reaction is catalyzed by pyruvate kinase (EC 2.7.1.4.). Phosphoenolpyruvate (PEP) synthesized following a literature procedure[13] is employed as a phosphate donor.

Thus the nucleotides remain in the cycle and are needed in only catalytical amounts. For economic reasons all six

enzymes are to be applied in immobilized form. Polyacrylamide, agarose and controlled pore glass are reported to give good results[12,14] as supports for these enzymes. In this work we have used the above mentioned activated CH-Sepharose as a support which offers the advantage of a very short immobilization procedure.

The reaction is performed in Tris-buffer containing manganese(II) chloride, magnesium chloride, potassium chloride, sodium azide, and bovine serum albumine (BSA). After addition of the 6-phosphate **11** (20 mM), acceptor (20 mM), PEP (21 mM), α-D-glucose 1,6-diphosphate (catalytical amount), and UTP (1 mM) the immobilized enzymes are suspended in the solution. The reaction vessel is placed on a shaking apparatus at 37 °C. The reaction turnover is quantified by measuring the amount of phosphate produced using a modified Fiske–Subarow method.[15] Isolation procedure and yields are the same as described above. In this case the low yield is probably due to the inhibitory effect of phosphate.[6]

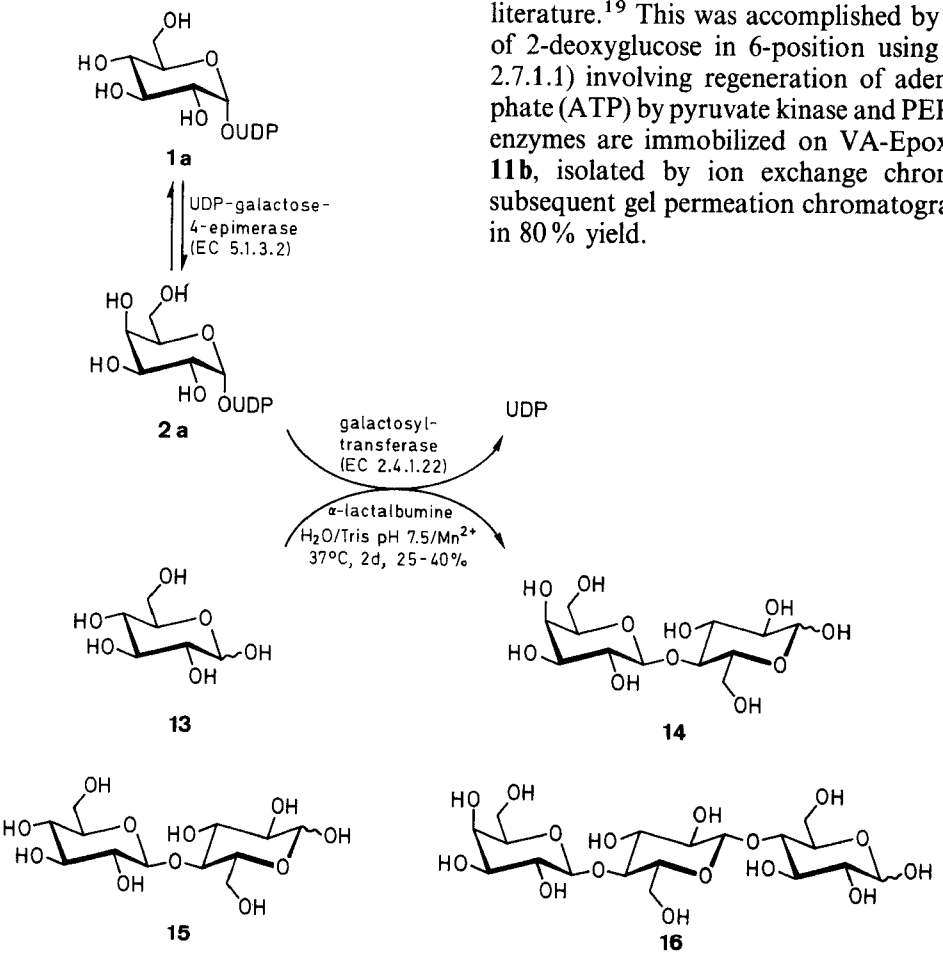
Among the glycosyltransferases the GalT is unique in its property to be specific for two different substrates. In addition to galactosylation of 2-acetamido-2-deoxy-glucose terminated oligosaccharides the GalT forms a complex with α-lactalbumine, called the lactose synthetase complex, which is now able to galactosylate glucose-terminated oligosaccharides.

In order to show that this reaction may also be performed on a preparative scale we have galactosylated glucose **(13)** to give lactose **(14)** and cellobiose **(15)** to give the trisaccharide **16** (Scheme 3). The former reaction is identical with the naturally occuring reaction for the synthesis of milk sugar. The synthesis is carried out in the same way as described above (see Scheme 1) with the only exception that α-lactalbumine is to be used instead of BSA. The enzymes are applied in free form since immobilization is expected to prevent the formation of the lactose synthetase complex.

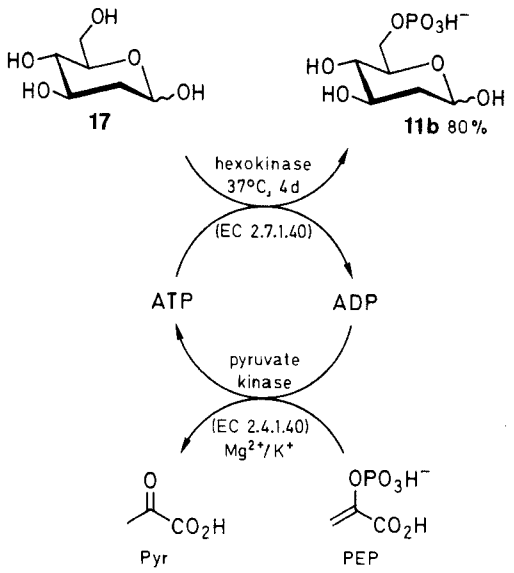
It is known that glycosyltransferases have a rather limited specificity with respect to the donor substrate. But apart from very few examples concerning variations of the nucleoside moiety[6] and of the sugar moiety[16] the donor substrate specifity of the GalT is not thoroughly explored.

We discovered that UDP-2-deoxy-galactose **(2b)** serves as a donor in the acetamidolactose synthetase reaction.[17] It is known that the 2-deoxy sugars **1b**, **11b** and **12b** (Scheme 2) are substrates of the respective enzymes used in the cycle.[18] Thus, **2b** is generated in situ starting from 2-deoxyglucose 6-phosphate **(11b)** via the very labile 2-deoxy-1-phosphate **12b** and the UDP derivative **1b**. The subsequent glycosylation step onto 2-acetamido-2-deoxyglucopyranose gives the 2′-deoxy analogue of acetamidolactose **4b**. Again exclusively the β(1→4) linkage is formed and no byproducts could be detected by NMR.

The starting material **11b** was synthesized before similar to a synthesis of glucose 6-phosphate given in the literature.[19] This was accomplished by phosphorylation of 2-deoxyglucose in 6-position using hexokinase (EC 2.7.1.1) involving regeneration of adenosine-5′-triphosphate (ATP) by pyruvate kinase and PEP (Scheme 4). The enzymes are immobilized on VA-Epoxy.[10] Compound **11b**, isolated by ion exchange chromatography and subsequent gel permeation chromatography, is obtained in 80% yield.



Scheme 3



Scheme 4

Activated CH-Sepharose 4B was purchased from Pharmacia, VA-Epoxy from Riedel-de-Haën and the enzymes from Sigma. Enzyme immobilization on activated CH-Sepharose 4B was performed following the literature procedure[11] whereas immobilization on VA-Epoxy was a slight modification of the recommended one[10] and is given below. NMR spectra were recorded on a Bruker WM 770 or WM 400 spectrometer, mass spectra on a double focussing 70–250 S (VG Analytical). All reactions were performed in degassed twice distilled H_2O.

Galactosylation of GlcNAc-Terminated Sugars Using the Two-Enzyme System:

The reaction was carried out in Tris buffer (100 mM, pH 7.5, 20 mL). After adding acceptor **3a**, **5**, **7**, or **9** (400 μmol, 20 mM) and UDP-glucose (258 mg, 400 μmol) the pH was readjusted. $MnCl_2$ (13 mg, 100 μmol) and bovine serum albumine (BSA) (10 mg) were dissolved and the enzymes were added in either free or immobilized form. (UDP-galactose-4-epimerase (yeast) 5 U, galactosyltransferase 2 U (bovine colostrum); approximate values). The reaction was maintained at 37°C and, in case that immobilized enzymes were used, placed on a shaking apparatus. The reaction was followed by TLC ($PrOH/AcOH/H_2O$, 85:12:3). The turnover was quantified by determing the amount of phosphate produced using a modified Fiske–Subarow method.[14]

After approximately 50% conversion the reaction was stopped by filtration of the immobilized enzymes. The mixture was worked up by anion exchange (Dowex-Cl) and the products **4a**, **6**, **8** and **10** were purified by gel permeation chromatography (Bio-Gel P2, 2 × 108 cm). Yields and selected spectroscopic data are listed in Table 1.

Galactosylation of GlcNAc-Terminated Sugars with Cofactor Regeneration:

Acceptors **3a**, **5**, **7**, or **9** (400 μmol, 20 mM), KCl (55 mg, 740 μmol), PEP (87 mg, 420 μmol), glucose 1,6-diphosphate (0.05 mg, catalytic), and UTP (56 mg, 10 μmol) were dissolved in Tris-buffer (100 mM, pH 7.5, 20 mL). The pH was readjusted, $MgCl_2$ (20 mg, 200 μmol) and $MnCl_2$ (16 mg, 100 μmol) were added and the solution was degassed with helium. BSA (0.5%) and the immobilized enzymes were added (phosphoglucomutase (rabbit muscle) 50 U, UDP-glucose-pyrophosphorylase (bovine liver) 10 U, inorganic pyrophosphatase (yeast) 30 U, UDP-galactose-4-epimerase (yeast) 5 U, galactosyltransferase (bovine colostrum) 2 U, pyruvate kinase (rabbit muscle) 50 U, approximate values). Control of turnover and work up were performed as described above. After filtering off the enzymes were ready for another reaction cycle.

Galactosylation of Glucose-Terminated Sugars:

The reaction was carried out exactly as described above using the two-enzyme system with the only alteration that BSA was replaced by α-lactalbumine. Glucose and cellobiose are thus galactosylated to give **14** and **16**, respectively. Yields and selected spectroscopic data are listed in Table 1.

Immobilization of Hexokinase and Pyruvate Kinase on VA-Epoxy:

The immobilization was performed in Hepes buffer (0.3 M, pH 7.5) containing $MgCl_2$ (30 mM), adenosine 5′-diphosphate (10 mM), glucose (25 mM), and pyruvate (25 mM). After degassing the solution VA-Epoxy (0.5 g) was suspended in buffer and vacuum was applied to remove gas bubbles in the pores. Surplus solution was removed so that the support had a doughy consistence. Enzymes (1000 U each) were added and the reaction was maintained on a shaking apparatus. After 2 d the immobilized enzymes were filtered off and washed with buffered solution.

Synthesis of 2-Deoxy-D-glucopyranosyl 6-Phosphate (11b):

2-Deoxyglucose (**17**, 0.4 g, 2.4 mol), phosphoenolpyruvate (0.54 g, 2.6 mmol), $MgCl_2$ (95 mg, 0.96 mmol), KCl (0.3 g, 4 mmol), and ATP (24 mg, 40 μmol) were dissolved in bidistilled H_2O (40 mL). The pH-value was adjusted to 7.5, the solution was degassed with helium and immobilized hexokinase and pyruvate kinase were added. The reaction was maintained at 37°C on a shaking apparatus. After 4 d TLC ($PrOH/EtOH/H_2O$, 5:3:2) indicated total conversion of educt. The enzymes were filtered off and the solution was applied to a DEAE-cellulose column (3 × 30 cm, previously equilibrated with 30 mM NH_4HCO_3). After running the column with H_2O (200 mL) a linear gradient of NH_4HCO_3 (0–0.1 mM, total volume 2 L, 2 mL/min) was applied. 2-Deoxyglucose 6-phosphate **(11b)** was eluted at approximately 30 mM NH_4HCO_3. Product containing fractions were combined and most of the NH_4HCO_3 was removed by repeated lyophilization. A final purification step was performed on Bio-Gel P2 (2 × 108 cm). Yield: 0.3 g (80%; diammonium salt).

^1H NMR (D_2O, internal standard: HDO): a) α-form: $\delta = 5.55$ (d, $J_{1,2a} = 3.0$ Hz, H-1), 2.31 (dd, $J_{1,2e} < 1.5$ Hz, $J_{2e,3} = 5.0$ Hz, H-2e), 1.90 (ddd, $J_{2a,2e} = 13.0$ Hz, $J_{2a,3} = 12.5$ Hz, H-2a).
b) β-form: $\delta = 5.13$ (d, $J_{1,2a} = 9.5$ Hz,H-1), 2.44 (dd, $J_{1,2e} < 1.5$ Hz, $J_{2e,3} = 5.0$ Hz, H-2e), 1.71 (ddd, $J_{2a,2e} = 11.5$ Hz, $J_{2a,3} = 11.0$ Hz, H-2a).

Synthesis of *O*-(2-Deoxy-β-D-galactopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose (4b):

The synthesis is performed as described above using cofactor regeneration. Acceptor **3a** is used and 2-deoxyglucose 6-phosphate **(11b)** instead of glucose 6-phosphate. Yield: 20 mg (40%).

^{13}C NMR (free sugar **4b**; 63 MHz,H_2O, internal standard: MeCN): $\delta = 173.31$ ($NHCOCH_3$), 99.42 (C-1′), 93.93 (C-1, β-form), 89.59 (C-1, α-form), 77.87 (C-4, α-form), 77.44 (C-4, β-form), 74.65 (C-5′), 73.79 (C-5, β-form), 71.48 (C-3, β-form), 69.17 (C-5, α-form), 68.30 (C-3, α-form), 66.72 (C-3′), 65.73 (C-4′), 60.43 (C-6′), 59.18 (C-6), 55.29 (C-2, β-form), 52.80 (C-2, α-form), 32.54 (C-2′), 21.25 ($NHCOCH_3$, β-form), 20.97 ($NHCOCH_3$, α-form).

^1H NMR (Heptaacetate of **4b**, acetone-d_6,internal standard: acetone-d_5): $\delta = 7.15$ (d, $J_{NH,2} = 9.4$ Hz, NH, α-form), 7.11 (d, $J_{NH,2} = 9.4$ Hz, NH, β-form), 6.06 (d, $J_{1e,2} = 3,6$ Hz, H-1, α-form), 5.77 (d, $J_{1a,2} = 8.8$ Hz, H-1, β-form), 5.23 (m, 1 H, H-3), 5.23 (m, 1 H, H-4′), 5.04 (ddd, $J_{2e',3'} = 3.2$ Hz, $J_{3',4'} = 5.0$ Hz, 1 H, H-3′), 4.83 (dd, $J_{1',2a'} = 9.6$ Hz, $J_{1',2e'} = 2.2$ Hz, 1 H, H-1′), 4.34 (dd, $J_{6a,6b} = 12.2$ Hz, 1 H, H-6a), 4.33 (ddd, $J_{2,3} = 10.4$ Hz, H-2, α-form), 4.23 (dd, 1 H, H-6b), 4.20 (dd, $J_{5',6a'} = 3.6$ Hz $J_{6a',6b'} = 12.2$ Hz, 1 H, H-6a′), 4.11 (dd, $J_{5',6b'} = 2,0$ Hz, 1 H H-6b′), 4.07 (m, H-2, β-form), 4.00 (ddd, $J_{4',5'} = 5.6$ Hz, 1 H, H-5′) 3.95 (t, $J_{3,4} = J_{4,5} = 9.6$ Hz, 1 H, H-4), 3.83 (ddd, $J_{5,6a} = 4.4$ Hz $J_{5,6b} = 2,2$ Hz, 1 H, H-5), 1.95 (m, 1 H, H-2e′), 1.76 (ddd $J_{2a',2e'} = 12.4$ Hz, $J_{2a',3'} = 12.5$ Hz, 1 H, H-2a′).
FAB-MS (free sugar **4b**): $m/z = 368$ ($M^+ + 1$), 390 ($M^+ + Na$).

(1) For preliminary communications cf. Ref. 7 and 16.
(2) Lemieux, R.U. *Chem. Soc. Rev.* **1978**, *7*, 423.
Schmidt, R.R. *Angew. Chem.* **1986**, *98*, 213; *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 212.
Ogawa, T.; Yamamoto, H.; Nukada, T.; Kitajima, T.; Sugimoto, M. *Pure Appl. Chem.* **1984**, *56*, 779.
Paulsen, H. *Angew. Chem.* **1990**, *102*, 851; *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 823.
(3) Drueckhammer, D.G.; Hennen, W.J.; Pederson, R.L.; Barbas, C.F.; III; Gautheron, C.M.; Krach, T.; Wong, C.-H. *Synthesis* **1991**, 499.
Stiller, R.; Thiem, J. In *Jahrbuch Biotechnologie*; Präve, P., Schlingmann, M., Crueger, W., Esser, K., Tauer, R., Wagner, F., Eds.; Carl Hanser Verlag: München, 1990; Vol. 3, p 101.
Toone, E.J.; Simon, E.S.; Bednarski, M.D.; Whitesides, G.M. *Tetrahedron* **1989**, *45*, 5365.
(4) Nunez, H.A.; Barker, R. *Biochemistry* **1980**, *19*, 489.
(5) Graßl, M. In *Methoden der enzymatischen Analyse*; Bergmeyer, H.U., Ed.; Verlag Chemie: Weinheim, 1970; Vol. 2, p 2071.
(6) Babad, H.; Hassid, W.Z. *J. Biol. Chem.* **1966**, *241*, 2672.
(7) Thiem, J.; Treder, W.; Wiemann, T. In *Dechema Biotechnology Conferences*; Behrens,. D., Ed.; Verlag Chemie: Weinheim, 1988; Vol. 2, p 189.
Thiem, J.; Wiemann, T. *Angew. Chem.* **1990**, *102*, 78; *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 80.
(8) Cowley, D.E.; Hough, L.; Peach, C.M. *Carbohydr. Res.* **1971**, *19*, 231.
Spinola, M.; Jeanloz, R.W. *J. Biol. Chem.* **1970**, *245*, 4158.
Kunz, H.; Waldmann, H. *Angew. Chem.* **1985**, 97, 885; *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 883.
(9) Pollitt, R.J.; Pretty, K.M. *Biochem. J.* **1974**, *141*, 141.
(10) Burg, K.; Mauz, O.; Noetzel, S.; Sauber, K. *Angew. Makromol. Chem.* **1988**, *157*, 105.
Product information: Riedel-de Haën: Seelze, 1987.
(11) *Affinity Chromatography, Principles and Methods*; Pharmacia Fine Chemicals: 1983.
(12) Wong, C.-H.; Haynie, S.L.; Whitesides, G.M. *J. Org. Chem.* **1982**, *47*, 5416.
(13) Hirschbein, B.L.; Masenod, F.P.; Whitesides, G.M. *J. Org. Chem.* **1982**, 47, 3765.
(14) Thiem, J.; Treder, W. *Angew. Chem.* **1986**, *98*, 1100; *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1096.
David, S.; Augé, C. *Pure Appl. Chem.* **1987**, *59*, 1501.
(15) Cooper, T.G. *Biochemische Arbeitsmethoden*; Walter de Gruyter: Berlin, 1981.
(16) Berliner, L.J.; Robinson, R.D. *Biochemistry* **1982**, *21*, 6340.
Palcic, M.M.; Hindsgaul, O. *Glycobiology* **1991**, *1*, 205.
(17) Thiem, J.; Wiemann, T. *Angew. Chem.* **1991**, *103*, 1184; *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1163.
(18) Biely, P.; Bauer, S. *Collect. Czech. Chem. Commun.* **1967**, *32*, 1588.
Druzhinina, T.N.; Kusov, Y.Y.; Shibaev, V.N.; Kochetkov, N.K.; Biely, P.; Kucar, S.; Bauer, S. *Biochim. Biophys. Acta* **1975**, *381*, 301.
(19) Bednarski, M.D.; Chenault, H.K.; Simon, E.S.; Whitesides, G.M. *J. Am. Chem. Soc.* **1987**, *109*, 1283.