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Practical Enzyme-Based Syntheses of Uridine 5′-Diphosphogalactose and Uridine 5′-Diphospho-*N*-acetylgalactosamine on a Gram Scale[1]

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Practical enzyme-based routes for the syntheses of uridine 5′-diphosphogalactose (UDP-Gal) and uridine 5′-diphospho-*N*-acetylgalactosamine (UDP-GalNAc) on millimole scales have been developed. The activity of galactokinase (EC 2.7.1.6) in crude enzyme extracts from galactose-adapted yeast, coupled to a regenerating system for ATP, provides convenient and economical access to galactose-α-1-phosphate (Gal-1-P) and galactosamine-α-1-phosphate (GalN-1-P). The transfer of UMP to the sugar-1-phosphates was also accomplished enzymatically by Gal-1-P uridyltransferase (EC 2.7.7.12) using uridine 5′-diphosphoglucose (UDP-Glc) as the UMP donor. UDP-Glc was in turn regenerated in situ from glucose-1-phosphate and UTP using UDP-Glc pyrophosphorylase (EC 2.7.7.9). The only chemical step in the sequence was the acetylation of UDP-GalN to afford UDP-GalNAc using *N*-acetoxysuccinimide. The moderate overall yields (43% and 34% for UDP-Gal and UDP-GalNAc from Gal-1-P and GalN-1-P, respectively) were compensated by the straightforward preparation of the starting materials, UTP and the corresponding sugar-1-P.

Introduction

Recent decades have seen an explosive advance in the fields of carbohydrate biology and glycobiology. The biological importance of complex carbohydrates has become a topic of broad interest in the biological sciences. These research activities have also sparked a renaissance in carbohydrate chemistry by creating an increased demand for well-defined carbohydrates. In response, numerous sophisticated chemical strategies have been developed for the stereocontrolled synthesis of oligosaccharides and glycoconjugates.[4-14] It has become obvious that the biological function of carbohydrates greatly exceeds their roles as intermediates in energy storage and as structural elements in cells. Special interest has been focused on their relevance in biological recognition phenomena: for example, they present specific molecular binding sites for bacterial and viral adhesion on cell surfaces[15-19] and they are involved in cell–cell signaling during growth and differentiation.[20,21]

One of the most difficult steps in the "classical" chemical synthesis of well-defined oligosaccharides has remained the stereospecific formation of the glycosidic linkage between monosaccharide units. The enzymatic formation of the glycosidic linkages, employing glycosyltransferases of the Leloir pathway,[22-27] is now recognized to be a useful alternative to chemical glycosidation methods.[4-14] As these enzymes exhibit both stereo- and regioselectivity, protection/deprotection and activation steps to direct the stereochemistry of the glycosidic bond can be circumvented.

Applications of glycosyltransferases to preparative synthesis have been hampered by (i) the limited availability of the enzymes and (ii) the high expense of the commercially available nucleoside phosphate sugars, which serve as activated sugar donors for these enzymes. Molecular cloning techniques are now providing access to an increasing number of glycosyltransferases,[28-31] and a range of enzymes may soon be available as tools in carbohydrate chemistry.

We have undertaken a program to develop practical, gram-scale syntheses of the eight nucleoside phosphate sugars used by mammalian glycosyltransferases.[32-35] To date we have reported methods for the syntheses of uridine 5′-diphosphoglucose (UDP-Glc),[36] cytidine 5′-mono-

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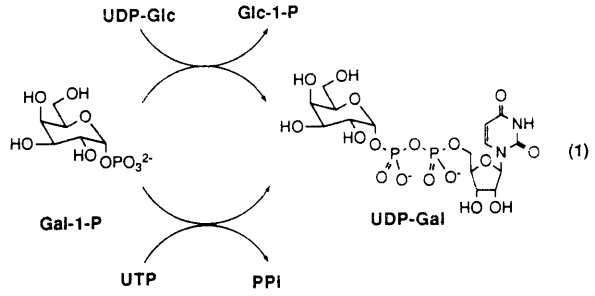
phospho-*N*-acetylneuraminic acid (CMP-Neu-5-Ac),[37] uridine 5′-diphosphoglucuronic acid (UDP-GlcUA),[38] guanosine 5′-diphosphomannose (GDP-Man),[36] guanosine 5′-diphosphofucose (GDP-Fuc),[39] and uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc).[40] Uridine 5′-diphosphogalactose (UDP-Gal) has been generated as intermediate in the enzymatic synthesis of *N*-acetyllactosamine.[41] We have now completed the series of activated sugars used by mammalian glycosyltransferases and report in this paper convenient enzyme-based syntheses of UDP-Gal and uridine 5′-diphospho-*N*-acetylgalactosamine (UDP-GalNAc). UDP-Gal[42,43] and UDP-GalNAc[44-48] have been prepared previously by various enzymatic and chemoenzymatic methods. Most of these procedures have been performed on small (analytical) scale and/or have been impractical for use in a synthetic chemical laboratory. Although a potentially useful enzymatic synthesis of UDP-GalNAc has been already reported by Maley,[49,50] we considered it worthwhile to develop a modified approach based on readily available starting materials.

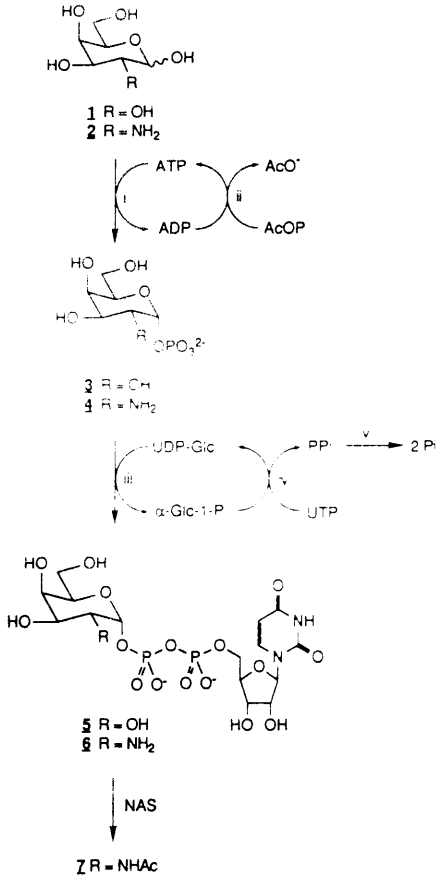
Results and Discussion

Biosynthesis and Enzymatic Strategy. UDP-Gal occurs in living organisms as an intermediate in the catabolic metabolism of D-galactose (Gal).[51] It is formed either by a uridyl transfer reaction from uridine 5′-diphosphoglucose (UDP-Glc) to α-D-galactose-1-P (Gal-1-P), a product of the enzymatic phosphorylation of Gal mediated by galactokinase (EC 2.7.1.6) or by a condensation between Gal-1-P and UTP catalyzed by UDP-galactose pyrophosphorylase (UDP-Gal Pase, EC 2.7.7.10) (eq 1).[52,53]



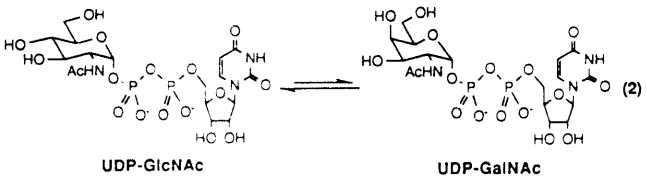
(1)

Scheme I. Enzyme-Based Synthesis of UDP-Galactose and UDP-*N*-Acetylgalactosamine[a]



[a] (i) Galactokinase, EC 2.7.1.6; (ii) acetate kinase, EC 2.7.1.40; (iii) galactose-1-phosphate uridyltransferase, EC 2.7.7.12; (iv) UDP-glucose pyrophosphorylase, EC 2.7.7.9; (v) pyrophosphatase, Ec 3.6.1.1. NAS is *N*-acetoxysuccinimide.

A subsequent isomerization of UDP-Gal at position 4 affords UDP-Glc, which can be catabolized further by enzymes of the glucose metabolism. In contrast, UDP-GalNAc is formed exclusively by epimerization of UDP-GlcNAc (eq 2) catalyzed by UDP-GlcN 4-epimerase (EC 5.1.3.7).[54,55]



(2)

Our enzymatic strategy for the synthesis of UDP-Gal and UDP-GalNAc was directed by the limited availability, narrow substrate specificity, and unfavorable equilibria of the enzymes involved in these pathways. For example, UDP-Glc 4-epimerase (EC 5.1.3.2) catalyzes the equilibrium between UDP-Glc and UDP-Gal in favor of UDP-Glc (K_{eq} = 3.0),[56] and no driving reaction is obvious to manipulate the equilibrium without consuming UDP-Gal.[57]

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(57) We had previously[41] prepared UDP-Gal on 80-mmol scale by isomerization from UDP-Glc using UDP-Glc 4-epimerase. The compound was, however, generated in situ and consumed immediately for the synthesis of *N*-acetyllactosamine.

UDP-Gal Pase[53] and UDP-GlcN 4-epimerase[45,46] are neither commercially available nor easy to prepare from biological sources. Consequently, we decided to synthesize UDP-Gal via the biosynthetic route: (i) phosphorylation of Gal to Gal-1-P by ATP using galactokinase and (ii) UMP transfer from UDP-Glc to Gal-1-P catalyzed by Gal-1-P uridyltransferase. We hoped also to employ these enzymes for an analogous preparation of UDP-GalNAc, since UDP-GlcN 4-epimerase, the enzyme primarily responsible for the biosynthesis of UDP-GalNAc, is not readily available (Scheme I). In fact, it was necessary to carry out the transformations using GalN, since GalNAc is not accepted as a substrate by either galactokinase or Gal-1-P uridyltransferase.[56]

Preparation of Gal-1-P (3) and GalN-1-P (4). The initial activation step in the metabolism of Gal (1) is the direct phosphorylation at the anomeric center catalyzed by galactokinase (EC 2.7.1.6) using ATP. This reaction yields Gal-α-1-P.[56] The reaction is thermodynamically favorable (K_{eq} = 26)[58] and, because it is strictly stereo- and regioselective, suitable as a convenient alternative to chemical methods of phosphorylation.

Previous preparations of **3**[44] and **4**[47] used galactokinase and stoichiometric amounts of ATP. Such approaches are therefore of only limited practical use for large-scale synthesis. As a consequence of the considerable price of purified galactokinase,[59] we investigated the use of crude enzyme extracts from two commercially available, Gal-adapted yeasts, *Klyveromyces fragilis* and *Candida pseudotropicalis*, as alternative sources for galactokinase. In principle, extracts from both strains, grown on Gal to induce the Gal-related enzymes, might be practical substitutes for the purified enzyme. In our hands, the extracts from *K. fragilis* provided superior results: about 5 units of galactokinase could be extracted from 1 g of the dried yeast cells, resulting in a 60% reduction in the cost for enzyme.

Two approaches were explored in the galactokinase-catalyzed phosphorylation of **1** and **2** to regenerate ATP from ADP. One was a coupled enzymatic reaction sequence leading from D-(−)-3-phosphoglyceric acid (PGA) via 2-phosphoglyceric acid to phosphoenol pyruvate (PEP).[60] PEP, in turn, transfers a phosphoryl group to ADP in a reaction catalyzed by pyruvate kinase (PK, EC 2.7.1.40).[60] We refer to this method of regenerating ATP from ADP as the 3-PGA/PK system. The second was the regenerating system using acetate kinase (AK, EC 2.7.2.1) and acetylphosphate (AcOP) as donor substrate (referred to here as the AcOP/AK system).[61] The results obtained for the enzymatic phosphorylation of **1** and **2** using purified galactokinase and crude enzyme extracts from *K. fragilis* coupled with these two regenerating systems for ATP from ADP are summarized in Table I.

Commercial, purified galactokinase was successfully applied to the preparation of Gal-1-P (**3**) from Gal (**1**). No GalN-1-P (**4**) could, however, be isolated from experiments with GalN (**2**), even when 5 units of enzyme was used. By contrast, the enzyme extracts from *K. fragilis* were able to catalyze the phosphorylation of both **1** and **2**. We did not identify the enzyme responsible for conversion of **2** to **4** and do not know if it is simply a galactokinase with a broader specificity than the commercially available one or if it is a different kinase specific for GalN. Compounds **3** and **4** could be prepared and isolated on a 18.5-mmol and 6-mmol scale, respectively, employing extracts from 5 g of dried yeast cells. Although the 3-PGA/PK system is more convenient to use for in situ regeneration of ATP than the AcOP/AK system—because AcOP must be freshly prepared before each reaction and 3-PGA is commercially available—we found the regeneration with AcOP to be more efficient. In addition, during reactions with **2** and 3-PGA/PK the color of the reaction mixtures turned slowly from yellow to brown as the products decomposed partially.[62]

Table I. Scale and Yields for the Enzymatic Phosphorylation of Gal and GalN

sugar	enzyme[a]	phosphoryl donor[b]	isolated sugar-1-P, mmol (yield, %)[c]
Gal	GK	AcOP	6.5 (53)
Gal	GK	3-PGA	3.5 (28)
Gal	*K. f.*	AcOP	18.5 (74)
Gal	*K. f.*	3-PGA	2 (17)
GalN	GK	AcOP	–[d]
GalN	GK	3-PGA	–[d]
GalN	*K. f.*	AcOP	6 (50)
GalN	*K. f.*	3-PGA	–[d]

[a] GK = galactokinase (EC 2.7.1.6), 5 units; *K. f.* = cell extracts of *Klyveromyces fragilis* obtained from 5 g of dried yields cells. [b] AcOP = disodium acetylphosphate; 3-PGA = D-(−)-3-phosphoglyceric acid. [c] Overall yield of isolated material (barium salt, based on Gal or GalN) after 48 h of reaction, as determined by ^{1}H and ^{31}P NMR. [d] Yield less than 5%.

Enzymatic Synthesis of UDP-Gal (5). The coupling of **3** and a UMP moiety involved UDP-Glc as UMP donor and was catalyzed by Gal-1-P uridyltransferase. UDP-Glc was present only in catalytic amounts and was regenerated in situ from Glc-1-P and UTP by UDP-Glc Pase (Scheme I). The hydrolysis of the pyrophosphate released by pyrophosphatase was employed to drive the enzymatic sequence. We emphasize that **3** and UTP are the only starting materials required in this system.

The reaction mixture was buffered at pH 8.4, which is optimum for Gal-1-P uridyltranferase and gave acceptable activities for UDP-Glc Pase and inorganic PPase.[56] The reaction system had to be optimized in respect to the concentration of Mg^{2+} ions: Mg^{2+} is essential for the activity of inorganic PPase[56] but inhibits Gal-1-P uridyltransferase[63] and promotes the nonspecific hydrolysis of the product.[49] We investigated the influence of Mg^{2+} ions on the reaction system in concentrations between 2 and 100 mM. The range between 5 and 10 mM provided optimum conditions for the interaction of the enzymes involved without significant hydrolysis of the UDP-sugars. The addition of 2-mercaptoethanol to the reaction buffer slightly enhanced the rate of reaction, since Gal-1-P uridyltransferase is stimulated by sulfhydryl agents.[63] Although we considered enzyme extracts from Gal-adapted yeast as an alternative source for Gal-1-P uridyltransferase, we concluded that they were not practical for use in a chemical laboratory: in addition to the desired Gal-1-P uridyltransferase, the extracts also contain UDP-Glc 4-epimerase. It would be necessary to separate this epimerase activity from the extracts by additional purification steps to avoid the isomerization of UDP-Gal to UDP-Glc.[43]

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Chemoenzymatic Synthesis of UDP-GalNAc (7). Gal-1-P uridyltransferase is active with **4** as substrate but not with GalNAc-1-P.[56] Therefore our strategy to synthesize UDP-GalNAc was based on the enzymatic preparation of UDP-GalN (**6**), followed by a chemical acetylation to afford UDP-GlcNAc (**7**). The experimental procedure used to prepare UDP-GalN was analogous to that used for UDP-Gal but required some modifications: Since the value of K_m of GalN-1-P for Gal-1-P uridyltransferase is significantly higher (12 mM) than that of Gal-1-P (0.4 mM),[64] the GalN-1-P had to be present in higher concentrations than Gal-1-P. The reaction rate with GalN-1-P is only ~5% relative to that of the natural substrate Gal-1-P,[64] and therefore more enzyme was needed and extended reaction times were required.

The acetylation of **6** to afford **7** was performed in situ on the crude reaction mixture, without isolation of the intermediate UDP-GalN. We reinvestigated acetylation procedures using acetic anhydride[45,49,50] that have been applied previously on small scale to this class of compounds. In our hands, these approaches led to nonquantitative reaction and resulted in a partial decomposition of the UDP-sugars when the procedure was scaled up to quantities on gram scale. Superior results were obtained by using *N*-acetoxysuccinimide (NAS) in water/THF.[65] These procedures resulted in quantitative acetylation of **6** to **7** and no formation of side products.

Conclusions

The enzyme-based syntheses of UDP-Gal (**5**) and UDP-GalNAc (**7**) described in this paper present practical alternatives to chemical procedures.[4-14] Compounds **2** and **3** have been obtained in a straightforward, affordable manner on a multigram scale using crude enzyme extracts from Gal-adapted yeast and an AcOP/AK system to regenerate the phosphoryl donor, ATP, from ADP. UDP-Gal (**5**) and UDP-GalN (**6**) can be prepared in millimole quantities by an enzyme-catalyzed uridyl transfer from UDP-Glc to **3** and **4**, respectively, employing the activity of Gal-1-P uridyltransferase; these reactions are coupled in situ to a regenerating system for UDP-Glc (condensation of Glc-1-P and UTP catalyzed by UDP-Glc Pase) and driven thermodynamically by hydrolysis of the pyrophosphate to phosphate. We believe that the regeneration of UDP-Glc from Glc-1-P and UTP in situ by the catalysis of UDP-Glc Pase is more useful for preparations on a large scale than the use of stoichiometric amounts of UDP-Glc.[49,50] The final step in the synthesis of **7** is the chemical acetylation of **6**, which proceeds quantitatively without decomposition or side reactions using *N*-acetoxysuccinimide (NAS). This acetylation is performed in situ with the crude product generated in the enzymatic reaction.

The yields of UDP-Gal from Gal-1-P and UDP-GalNAc from GalN-1-P were 43% and 34%, respectively. These values are limited by the procedures required to obtain products of a high degree of purity from the complex reaction mixture. Although comparable yields are obtainable by the commonly used "morpholidate" procedure[66] (reacting the sugar-1-P with UMP-morpholidate), the practicability of the enzyme-based approach is enhanced by the ready availability of the starting material UTP[36] relative to UMP-morpholidate.

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Experimental Section

General Methods and Materials. Chemicals and solvents were reagent grade and were used without further purification. Water was distilled from glass in a Corning AG-1b still. Ion-exchange resins (Dowex 50W-X8, H^+ form, 100–200 mesh; AG1-X8, formate form, 100–200 mesh) were obtained from Biorad; media for gel filtration (Sephadex LH-20) were from Pharmacia. Activated charcoal (decolorizing, Norit 211) was received from Kodak. D-Gal was purchased from Aldrich, D-GalN·HCl and ATP (disodium salt) were from Sigma. D-(−)-3-Phosphoglyceric acid (containing ~2% 2,3-diphosphoglyceric acid) was obtained from US Biochemicals. UTP was afforded by chemical deamination from CTP, which was generated from CMP in a coupled reaction system using 3-PGA as phosphoryl donor as described previously.[36] A solution of disodium acetyl phosphate (~1 M) was freshly prepared before use from phosphoric acid (85%) and acetic anhydride.[61]

The enzymes (lyophilized preparations or crystalline suspensions in solutions of ammonium sulfate) and dried yeast cells were purchased from Sigma: acetate kinase (from *E. coli* EC 2.7.2.1, Sigma A-7779), enolase (from bakers' yeast, EC 4.2.1.11, Sigma E-6126) galactokinase (from Gal-adapted yeast, EC 2.7.1.6, Sigma G-0130), galactase-1-phosphate uridyltransferase (from galactose-adapted yeast, EC 2.7.7.12, Sigma G-4256), pyrophosphatase (from bakers' yeast, EC 3.6.1.1, Sigma I-4503), phosphoglycerate mutase (from rabbit muscle, EC 2.7.5.3, Sigma P-8252), pyruvate kinase (from rabbit muscle, EC 2.7.5.3, Sigma P-7768), UDP-glucose pyrophosphorylase (type X from bakers' yeast, EC 2.7.7.9, Sigma U-8501), galactose-adapted yeasts *Kluyveromyces fragilis* (ATCC No. 10022, Sigma YGA) and *Candida pseudotropicalis* (ATCC No., 2512 Sigma YCP). Commercial enzymes were not assayed; the reported activities refer to the activities stated by the manufacturer: 1 unit of activity converts 1 μmol of substrate to products per minute under defined assay conditions.

The enzyme-catalyzed reactions were performed in sealed Erlenmeyer flasks under argon at rt. Oxygen was removed from the buffers before use by bubbling a stream of argon through the stirred solution for 30 min. The reactions were monitored by TLC (silica gel 60 F-254; eluant for Gal-1-P, 1-propanol/water/acetic acid, 15/4/0.5, v/v/v; eluant for GalN-1-P, ethanol/1 M ammonium formate (pH 3.8), 7/3, v/v; eluant for UDP-Gal, UDP-GalN, and UDP-GalNAc, 1-propanol/ammonia/water, 6/3/2, v,v,v).

^{1}H NMR spectra were obtained in D_2O at 400 MHz, and the chemical shifts (δ) are reported in ppm and referenced to HOD at δ 4.8 ppm. ^{13}C NMR spectra in D_2O were measured at 100 MHz, and the chemical shifts are given relative to external dioxane set at δ 67.6 ppm. ^{31}P NMR spectra were performed at δ 121.49 MHz and referenced to external H_3PO_4 (85%) set at 0.0 ppm. The reported purities of the isolated products are based on internal standard quantification by ^{1}H NMR (internal standard dioxane) and ^{31}P NMR spectroscopy.

Gal-1-P (3). Galactokinase/AcOP/AK Method. To 125 mL of Tris/HCl buffer (10 mM, pH 7.5) containing $MgCl_2 \cdot 6H_2O$ (500 mg, 2.5 mmol) and 2-mercaptoethanol (48 mg, 0.6 mmol) in a 250-mL Erlenmeyer flask was added **1** (2.25 g, 12.5 mmol), $ATP \cdot Na_2$ (70 mg, 0.125 mmol), disodium acetyl phosphate (AcOP) solution (~1 M, 8 mL, 8 mmol), bovine serum albumin (125 mg), galactokinase (5 units), and acetate kinase (AK, 50 U). Additions of 5-mL portions of AcOP solution were repeated after 11, 25, and 36 h, and after each addition the pH of the reaction mixture was readjusted to 7.5 with 5 N KOH. After 48 h, $BaCl_2 \cdot H_2O$ (7.3 g, 30 mmol) was added and the solution was stirred at 4 °C for 6 h. The resulting precipitate (mainly barium phosphate) was removed by filtration. Addition of 1 volume of cold acetone to the clear filtrate yielded a cloudy precipitate, which was recovered by filtration after 24 h at 4 °C. The precipitate was washed with two 50-mL portions of a cold mixture of acetone/water (1/1, v/v) and 50 mL of acetone and dried in vacuo, yielding 4.39 g of product as a white powder. An aliquot was treated with ion-exchange resin (Dowex 50W-X8, H^+ form) and neutralized with NaOH. After evaporation to dryness at reduced pressure, the residue was dissolved in D_2O and investigated by NMR. The purity of Gal-1-P (barium salt) was estimated to be ~60% by NMR spectroscopy, using internal standards for quantification (53% yield based on

1):[67] ^{1}H NMR (D_2O, 400 MHz) δ 5.42 (dd, H1, J = 7.4, 3.6 Hz), 4.19 (ddd, H5, J = 7.1, 4.9, 1.0 Hz), 4.11 (dd, H4, J = 3.0, 1.0 Hz), 3.93 (dd, H3, J = 10.2, 3.4 Hz), 3.78 (ddd, H2, J = 1.7, 3.6, 10.2 Hz), 3.78 (m, H6a), 3.73 (dd, H6b, J = 11.7, 4.8 Hz); ^{13}C NMR (D_2O, 100 MHz) δ 94.7 (d, C1, J = 5.5 Hz), 71.9 (C5), 70.4 (C4), 70.3 (C3), 69.8 (d, C2, J = 6.2 Hz), 62.2 (C6).

Gal-1-P (3). Galactokinase/3-PGA/PK Method. The reaction was performed following the procedures described above, but instead of AcOP/AK the following were added: enolase (200 units), phosphoglycerate mutase (200 units), pyruvate kinase (PK, 300 units), and a solution of 3-PGA (disodium salt, pH 7.5) obtained from the corresponding barium salt (6.0 g, 18.7 mmol) by treatment with ion-exchange resin (H^+ form) and neutralization with NaOH. After 48 h Gal-1-P was recovered as barium salt (1.38 g, 28% yield based on **1**).

Preparation of Cell Extracts from *K. fragilis*. Lyophilized cells (2.5 g) were suspended in 20 mL of Tris/HCl buffer (50 mM, pH 7.5) containing 2-mercaptoethanol (5 mM) in a 50-mL, disposable, polyethylene centrifuge tube. Glass beads (acid washed; diameter between 0.25 and 0.30 mm, ~12 g) were added, and the sealed tube was vigorously agitated on a Vortex laboratory mixer for about 8 min with intermediate periods of cooling on ice. The supernatant liquid was decanted, the glass beads were washed with buffer (10 mL), and the washing solution was combined with the supernatant. Cell fragments were sedimented by centrifugation (8000*g*, 4 °C, 10 min) and discarded. To the supernatant liquid was added 1 mL of a solution of protamine sulfate (30 mg dissolved in 1 mL of water at 50 °C), and the solution was stirred at 4 °C for 15 min. The precipitate was removed by centrifugation (15000*g*, 4 °C, 15 min), and the supernatant (~25 mL) was dialyzed overnight against Tris/HCl buffer (10 mM, pH 7.5) containing 2-mercaptoethanol (5 mM). The protein concentration in the dialyzed extracts was determined to be ~15 mg/mL using a Coomassie dye protein assay according to the method of Bradford,[68] referencing the calibration curve to bovine serum albumin as standard protein. The activity of galactokinase in the extracts was estimated at ~1 units/mL (~0.07 units/mg protein).

Gal-1-P (3). Cell Extracts/AcOP/AK Method. To 400 mL of Tris/HCl buffer (10 mM, pH 7.5) containing 2-mercaptoethanol (5 mM) and $MgCl_2$ (20 mM) (buffer A) were added **1** (4.5 g, 25 mmol), ATP·Na_2 (300 mg, 0.54 mmol), AcOP solution (~1 M, 15 mL, 15 mmol), acetate kinase (200 units), and cell extracts obtained from 5 g of lyophilized yeast cells. Portions of AcOP solution (10 mL) were added after 8, 22, and 28 h, and after each addition the pH of the solution was readjusted to 7.5 with 5 N NaOH. After 48 h the reaction was quenched by addition of $BaCl_2$ (20.8 g, 100 mmol) and stirred at 4 °C for 6 h. The white precipitate was removed by filtration, and 1 volume of cold acetone was added, leading to a cloudy precipitate, which was recovered by filtration after 16 h at 4 °C. The residue was washed with a cold mixture of acetone/water (1/1, v/v, 150 mL) and acetone (50 mL). After the residue was dried at reduced pressure, 7.33 g of a white powder was obtained. This material was investigated by NMR spectroscopy after treatment with ion-exchange resin (H^+ form). The product was indistinguishable from authentic material: Gal-1-P (barium salt, ~85%, 74% yield).[67]

GalN-1-P (4). Cell Extracts/AcOP/AK Method. To 100 mL of buffer A were added GalN·HCl (2.5 g, 11.6 mmol, dissolved in 8 mL of water and adjusted to pH 7.5 with 5 N KOH before addition), ATP·Na_2 (75 mg, 0.135 mmol), AcOP solution (~1 M, 8 mL, 8 mmol), acetate kinase (5 units), and cell extracts from 5 g of yeast cells, prepared according to the procedures detailed above. Additional 5-mL portions of the AcOP solution were supplied after 8, 22, and 28 h. The reaction was stopped after 48 h by the addition of $BaCl_2$ (12.5 g, 60 mmol), and the product was isolated as described above for **3** as the barium salt (~80%, 2.87 g, 50% yield):[67] ^{1}H NMR (D_2O, 400 MHz) δ 5.72 (dd, H1, J = 6.2, 3.5 Hz), 4.11 (br t, H5, J = 6.2 Hz), 4.06 (dd, H3, J = 10.8, 3.1 Hz), 4.01 (br d, H4, J = 3.0 Hz), 3.73 (dd, H6a, J = 11.8, 7.0 Hz), 3.69 (dd, H6b, J = 11.8, 5.4 Hz), 3.51 (dt, H2, J = 10.8, 2.9 Hz); ^{13}C NMR (D_2O, 100 MHz) δ 93.08 (d, C1, J = 5.2 Hz), 72.90 (C5), 68.77 (C4), 67.08 (C3), 61.65 (C6), 51.75 (d, C2, J = 8.9 Hz).

UDP-Gal (5). A suspension of **3** (barium salt, prepared by using the cell extracts/AcOP/AK method from **1**, ~85%, 4.65 g, ~10 mmol) and 40 mL of water was stirred with 40 mL of ion-exchange resin (Dowex 50W-X8, H^+ form) for 20 min. The resin was removed by filtration and washed with three 20-mL portions of water, and the combined filtrates were neutralized with 5 N KOH. The solution was transferred into a flask with 800 mL of water containing $MgCl_2·6H_2O$ (800 mg, 4 mmol), UTP·$Na_3·2H_2O$ (~90%, 2.1 g, ~3.25 mmol), UDP-Glc·Na_2 (100 mg, ~0.2 mmol), and 2-mercaptoethanol (312 mg, 4 mmol). The pH of the solution was adjusted to 8.5 with 5 N KOH, and pyrophosphatase (500 units), UDP-Glc pyrophosphorylase (100 units), and Gal-1-P, uridyltransferase (50 units) were added. After 4, 8, and 20 h, 2.1-g portions of UTP·$Na_3·2H_2O$ (3.25 mmol) were added, respectively, and the pH of the mixture was readjusted to 8.5. After 48 h, the reaction was stopped by immersing the flask in a bath of boiling water for 3 min. The precipitated proteins were removed by centrifugation (8000*g*, 10 min). The supernatant liquid was applied to the top of a column of ion-exchange resin (AG1-X8, formate form, 6 cm × 20 cm), and the column was washed with 700 mL of water and 200 mL of 4 N formic acid. Compound **5** was eluted from the column in a gradient of 4 N formic acid and 4 N formic acid containing 0.5 M ammonium formate (1.5 L) at an ammonium formate concentration of ~0.25 M. The fractions containing UDP-Gal were combined and stirred with ~100 g of charcoal for 30 min. The charcoal was separated by filtration and washed with 800 mL of water. UDP-Gal was desorbed from the charcoal by washing with 400 mL of a mixture of water/ethanol/ammonia (50/45/5, v/v/v). Fine particles of charcoal were removed by filtration through Celite 545, and the clear filtrates were concentrated at reduced pressure to a volume of ~5 mL. Contaminants of formate were removed by passing through a gel filtration column (Sephadex LH-20, 1 cm × 90 cm) using water as eluent. The collected fractions containing UDP-Gal were combined and stirred with 15 mL of ion-exchange resin (Dowex 50W-X8, H^+ form). The resin was removed by filtration and washed with three 20-mL portions of water. The combined filtrates were neutralized with 1 N NaOH and concentrated in vacuo, yielding UDP-Gal (disodium salt, 2.52 g, 43% yield):[69] ^{1}H NMR (D_2O, 400 MHz) δ 7.91 (d, H6″, J = 8.1 Hz), 5.94 (d, H5″, J = 8.1 Hz), 5.87 (d, H1′, J = 4.2 Hz), 5.60 (dd, H1, J = 7.3, 3.6 Hz), 4.33 (m, H2′, H3′), 4.25 (m, H4′), 4.21 (m, H5′a), 4.16 (m, H5′b), 4.13 (t, H5, J = 6.3 Hz), 3.98 (d, H4, J = 3.2 Hz), 3.87 (dd, H3, J = 10.3, 3.3 Hz), 3.76 (dt, H2, J = 10.6, 3.3 Hz), 3.72 (dd, H6a, J = 11.8, 7.0 Hz), 3.68 (dd, H6b, J = 11.8, 5.3 Hz); ^{13}C NMR (D_2O, 100 MHz) δ 167.3 (C4″), 152.9 (C2″), 142.7 (C6″), 103.7 (C5″), 96.9 (d, C1, J = 6.85 Hz), 89.6 (C1′), 84.2 (d, C4′, J = 8.95 Hz), 74.8 (C3′), 73.0 (C5), 70.8, 70.4, 70.2 (C2′, C3, C4), 69.4 (d, C2, J = 8.65 Hz), 66.1 (d, C5′, J = 5.63 Hz), 62.1 (C6).

UDP-GalNAc (7). Enzymatic Preparation of UDP-GalN (6). The enzymatic synthesis of **6** followed the procedures described for **5**, with the exception of the following modifications: compound **4** (barium salt, prepared by using the cell extracts/AcOP/AK method from GalN, ~70%, 4.5 g, ~8 mmol) instead of Gal-1-P and UTP·$Na_3·2H_2O$ (~90%, 6.5 g, ~10 mmol) was supplied in four portions to the buffer solution (450 mL) over a period of 60 h. After 48 h, an additional quantity of Gal-1-P uridyltransferase (35 units) was added to the reaction mixture. The reaction was quenched after 72 h, and an analytical sample of **6** (disodium salt) was purified as described above for **5**: ^{1}H NMR (D_2O, 400 MHz) δ 7.92 (d, H6″, J = 8.1 Hz), 5.96 (d, H1′, J = 4.4 Hz), 5.94 (d, H5″, J = 8.1 Hz), 5.80 (dd, H1, J = 6.7, 3.5 Hz), 4.34 (m, H2′, H3′), 4.27 (m, H4′), 4.25 (m, H5′a), 4.18 (m, H5′b), 4.17 (m, H5), 4.03 (m, H3, H4), 3.76 (dd, H6a, J = 11.9, 7.2 Hz), 3.74 (dd, H6b, J = 11.9, 5.3 Hz), 3.45 (dd, H2, J = 10.9, 3.3 Hz); ^{13}C NMR (D_2O, 100 MHz) δ 167.3 (C4″), 152.8 (C2″), 142.5 (C6″), 103.5 (C5″), 94.6 (d, C1, J = 5.4 Hz), 89.5 (C1′), 85.0 (d, C4′, J = 9.1 Hz), 74.7 (C3′), 73.1 (C5), 70.5, 69.0, 68.1 (C2′, C3,

(67) The ^{1}H and ^{13}C NMR spectra were indistinguishable from authentic material (Sigma); ^{31}P NMR spectroscopy revealed that inorganic phosphate was present as a contaminant.

(68) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248.

(69) A gradient elution from the column of ion-exchange resin was essential for product purity; a batchwise elution with 4 N formic acid and 4 N formic acid containing 0.4 M ammonium formate resulted in UMP (~10%) as an impurity after all purification steps.

C4), 66.0 (d, C5′, J = 5.2 Hz), 61.8 (C6), 51.77 (d, C2, J = 9.1 Hz).

Chemical Acetylation of 6. The crude reaction mixture obtained from the preceding step was concentrated to 100 mL at reduced pressure, and *N*-acetoxysuccinimide[65] (~90%, 2.63 g, ~15 mmol), dissolved in a mixture of water/THF (30 mL, 1/1, v/v), was added to the stirred solution at rt. The pH of the reaction was controlled at ~7.5 by the addition of 5 N NaOH. After 7 h the pH was constant and the product purification was accomplished as described above for **5**. Compound **7** was obtained as the disodium salt (1.75 g, 34% yield), a white powder, which was indistinguishable by ^{1}H and ^{13}C NMR spectroscopy from authentic material: ^{1}H NMR (D_2O, 400 MHz) δ 7.94 (d, H6″, J = 8.1 Hz), 5.97 (d, H1′, J = 4.7 Hz), 5.95 (d, H5″, J = 8.1 Hz), 5.54 (dd, H1, J = 7.1, 3.3 Hz), 4.45–4.30 (m, H2′, H3′), 4.30–4.16 (m, H4′, H5′a, H5′b, H2, H5), 4.04 (br d, H4, J = 2.5 Hz), 3.96 (dd, H3, J = 11.0, 3.1 Hz), 3.78 (dd, H6a, J = 7.2, 11.8 Hz), 3.73 (dd, H6b, J = 5.2, 11.8 Hz), 2.08 (s, CH_3); ^{13}C NMR (D_2O, 100 MHz) δ 175.8 (C=O, Ac), 167.1 (C4″), 152.7 (C2″), 142.5 C6″), 103.5 (C5″), 95.5 (d, C1, J = 6.1 Hz), 89.4 (C1′), 84.1 (d, C4′, J = 9.1 Hz), 74.6 (C3′), 72.9 (C5), 70.5 (C2′), 69.2 (C4), 68.5 (C3), 65.9 (d, C5′, J = 5.6 Hz), 61.9 (C6), 50.62 (d, C2, J = 8.0 Hz), 23.0 (CH_3, Ac).