Gram-Scale Synthesis of Uridine 5'-Diphospho-N-acetylglucosamine: Comparison of Enzymatic and Chemical Routes[1]

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Practical chemoenzymatic and chemical routes to uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc) on a gram scale have been developed. The chemoenzymatic synthesis provided convenient access to glucosamine-6-phosphate and N-acetylglucosamine-6-phosphate (GlcNAc-6-P) in >10-mmol quantities. The condensation between GlcNAc-6-P and UTP was catalyzed by readily available crude enzyme extracts from dried cells of the yeast *Candida utilis* and afforded a 17% yield of UDP-GlcNAc from GlcNAc-6-P. The otherwise straightforward chemoenzymatic sequence was hampered by the need to purify the product from the final complex reaction mixture. The chemical synthesis of UDP-GlcNAc proceeded through five steps in an overall yield of 15% from pentaacetylglucosamine with the selective formation of tetraacetylglucosamine-α-1-phosphate as the key reaction.

Introduction

Oligosaccharides are currently a topic of intense interest in biochemistry.[5-8] Their synthesis remains a particularly challenging task.[9-11] We are exploring enzymatic approaches to this class of compounds, with a particular interest in minimizing the number of protection/deprotection steps.[8] One approach toward this goal involves the use of the glycosyltransferases of the Leloir pathway—enzymes that catalyze the regio- and stereoselective formation of glycosidic linkages in the biosynthesis of a variety of oligosaccharides and glycoconjugates.[7,12-14] To take full advantage of these glycosyltransferases, practical methods of preparing the nucleoside mono- and diphosphate sugars (the activated forms of the sugars used in this pathway) are required. We have previously described routes to CMP-Neu-5-Ac,[15] UDP-Glc,[16] GDP-Man,[16] UDPGluA,[16] UDP-Gal,[17] and GDP-Fuc.[18]

In this paper, we report two practical (gram-scale) syntheses of uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc). We have explored both chemoenzymatic and chemical routes to this compound; this paper describes and compares these routes. UDP-GlcNAc has previously been prepared by chemical synthesis,[19] enzymatic syn-

Scheme I. Biosynthesis of UDP-GlcNAc[a]
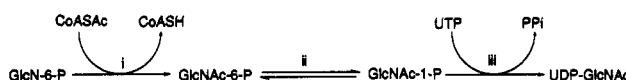
[a] (i) Glucosamine-6-phosphate N-acetyltransferase, EC 2.3.1.4; (ii) N-acetylglucosamine phosphomutase, EC 2.7.5.2; (iii) UDP-GlcNAc pyrophosphorylase, EC 2.7.7.23.

Scheme II. Chemoenzymatic Synthesis of UDP-GlcNAc
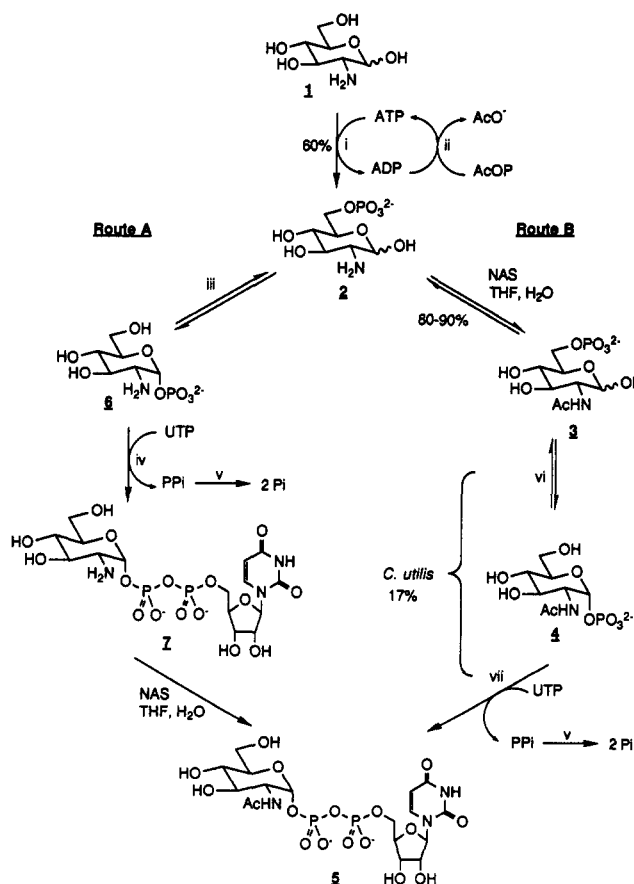
[a] (i) Hexokinase, EC 2.7.1.1; (ii) acetate kinase, EC 2.7.2.1; (iii) phosphoglucomutase, EC 5.4.2.2; (iv) UDP-Glc pyrophosphorylase, EC 2.7.7.9; (v) pyrophosphatase, EC 3.6.1.1; (vi) N-acetylglucosamine phosphomutase, EC 2.7.5.2; (vii) UDP-GlcN pyrophosphorylase, EC 2.7.7.23. NAS is N-acetoxysuccinimide.

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(3) NSERCC Centennial Scholar.

(4) NATO postdoctoral fellow, 1988–1989.

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thesis,[20-26] and fermentation processes,[27,28] albeit on submicromole-scale in most instances. Recently, a chemoen-

zymatic synthesis of UDP-GlcNAc has been published and demonstrated by preparation of submillimole quantities.[29]

Results and Discussion

Enzymatic Synthesis. None of the enzymes in the biosynthetic pathway of UDP-GlcNAc (Scheme I) are commercially available or easily prepared.[30-34] Rather than trying to clone and overexpress these enzymes, we explored the alternative enzyme-catalyzed sequences summarized in Scheme II. *N*-Acetylglucosamine-6-phosphate (GlcNAc-6-P) (3) is not accessible by the direct phosphorylation of *N*-acetylglucosamine (GlcNAc) using hexokinase (EC 2.7.1.1), since hexokinase does not accept GlcNAc as a substrate.[35] Compound 3 can, however, be prepared conveniently from glucosamine (GlcN) 1 in a two-step sequence: hexokinase-catalyzed phosphorylation of 1 by ATP leading to glucosamine-6-phosphate (GlcN-6-P) 2, followed by nonenzymatic acylation of 2 with *N*-acetoxysuccinimide (NAS).

We investigated two strategies for in situ regeneration of ATP in the phosphorylation of 1. We found the one that used acetyl phosphate to regenerate ATP from ADP[36,37] gave superior results relative to that based on the more stable, and therefore generally more useful, phosphoenol pyruvate (PEP).[36,37] Use of PEP resulted in decomposition of both 1 and 2, yielding intractable brown reaction products after workup.[38] Acetyl phosphate as the source of phosphate eliminated this decomposition, provided the reaction time was less than 5–6 h.[39] The rate

of phosphorylation we observed for 1 was only 1.4% that of glucose, 70% of that previously reported,[40] as measured by enzymatic and ^{31}P NMR assay. It is, however, still possible to take practical advantage of this minor side activity of hexokinase to obtain 2 in a gram scale, as hexokinase is commercially available and inexpensive.[41]

For the synthesis of UDP-GlcNAc 5 starting from 2 and UTP we investigated two different enzymatic routes (Scheme II), exploiting either the side activities of commercial enzymes (route A) or enzyme activities present in crude extracts from commercially available dried yeast cells (route B). In route A we intended to isomerize 2 to GlcN-1-P 6 using phosphoglucomutase (PGM, EC 5.4.2.2) and couple 6 in situ with UTP to give UDP-GlcN 7 in a reaction catalyzed by UDP-glucose pyrophosphorylase (UDP-Glc PPase, EC 2.7.7.9). The enzymatic cleavage of the pyrophosphate that is released by pyrophosphatase (PPase, EC 3.6.1.1) should act as the driving force of this reaction. Both PGM and UDP-GlcPPase are involved in the biosynthesis of UDP-Glc and were recently used in a hollow fiber enzyme reactor to prepare 7 in micromole quantities.[24] Reinvestigation of this strategy, however, showed that it is probably not economical for gram-scale preparations due to (i) the significant inhibition of the enzymes by 2 and UTP,[24] (ii) the low activity of PGM toward 2 (~1% relative to Glc-6-P) and for UDP-Glc PPase toward UDP-GlcN (~3% relative to UDP-Glc), and (iii) the high price of the enzymes.[42]

Faced with the shortcomings of route A, we investigated cell extracts from dried yeast cells of *Candida utilis* for desired enzyme activities. Cells of *C. utilis*, in combination with cells of fresh bakers' yeast, have been used previously for small-scale preparations of [β-^{32}P]-UDP-GlcNAc.[43] We employed a straightforward procedure for making the extracts using a laboratory mixer and glass beads to grind the cells (see the Experimental Section). These extracts, to which we added PPase, converted 3 and UTP to 5 with good activity; we used extracts from 10 g of dried yeast cells for 10-mmol-scale experiments. UTP and 3 in concentrations higher than 4 and 8 mM, respectively, resulted in a decrease in the rate of reaction. The accumulation of 4 during these experiments indicated that the decrease is probably a consequence of substrate inhibition of the UDP-GlcN PPase. To avoid this inhibitory effect, both substrates were added in portions over the course of the reaction. The extracts were also active with UTP and 2, yielding 7. The rate of reaction was, however, slow (an observation not in accord with previous results[44]).

To isolate 5 from the complex reaction mixture,[45] we investigated different purification procedures: (i) ad-

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(35) Enzyme assays at pH 7.5 contained GlcNAc, ATP, and hexokinase, and the reaction was monitored by ^{31}P NMR spectroscopy. GlcNAc is described as a competitive inhibitor for hexokinase. Colowick, S. In *The Enzymes*, 3rd ed.; Boyer, P., Ed.; Academic: New York, 1973; Vol. 9, p 1.

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(38) Examinations of the reactions of 1 and 2 with PEP and pyruvate at pH 7.5 in the absence of enzyme indicated that the interaction of 1 and 2 with pyruvate was responsible the nonenzymatic browning reaction. ^{1}H NMR analysis of the reaction products showed weak signals in the aromatic region (8.7 and 8.3 ppm), and we propose that the browning is probably due to an intramolecular condensation of an intermediate enaminoaldehyde, as given in eq i for 1 and pyruvate.


$$(i)$$
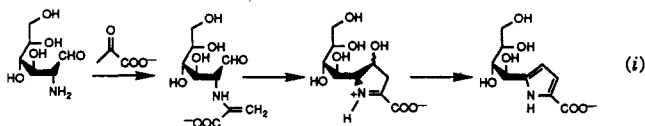
A related reaction between pyruvate and hydroxylamine derivatives has been described and implicated in the biosynthesis of siderophore aerobactin, see: Goh, C. J.; Szcepan, E. W.; Wright, G.; Menhart, N.; Honek, J. F.; Visvanatha, T. *Bioorg. Chem.* 1989, *17*, 13.

(39) Acetyl phosphate (AcOP) is unstable at neutral pH at room temperature, its half-life is 21 h in these conditions. A short reaction time can minimize the AcOP hydrolysis as well as the slow decomposition of GlcN and GlcN-6-P. The inorganic phosphate liberated in the AcOP hydrolysis can easily be eliminated by selective crystallization since its barium salt is insoluble in water unlike the barium salt of GlcN-6-P. The membrane-enclosed enzymatic catalysis (MEEC) technique (see: Bednarski, M. D.; Chenault, H. K.; Simon, E. S.; Whitesides, G. M. *J. Am. Chem. Soc.* 1987, *109*, 1283) dramatically slowed the rate of reaction and resulted in more degradation and lower yield.

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(41) The estimated cost (based on research-scale quantities from Sigma) for 1 unit of hexokinase is ~$0.005.

(42) The estimated cost for 1 unit of PGM is ~$0.04 and of 1 unit of UDP-Glc PPase is ~$0.3.

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(45) The reaction mixtures contained in addition to proteins, residual starting materials (UTP and sugar phosphates), UDP and UMP (resulting from hydrolysis of UTP), and phosphate (as byproduct of the reaction).

Scheme III. Chemical Synthesis of UDP-GlcNAc
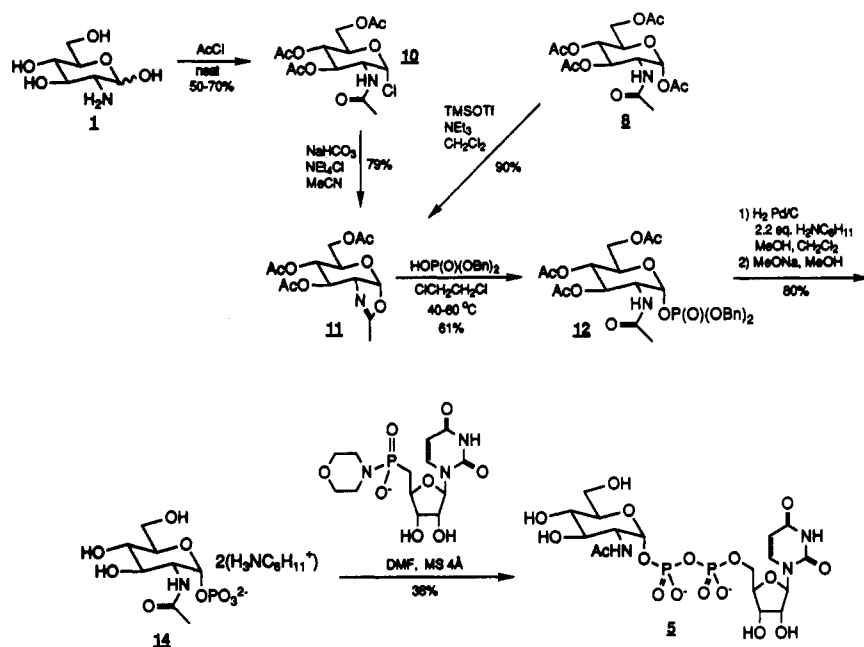
sorption/desorption on/from charcoal; (ii) ion-exchange chromatography on AG1-X8 (eluent: formate); (iii) ion-exchange chromatography on DE-52; and (iv) gel filtration on Sephadex LH-20. No single procedure was sufficient to achieve a high level of purification. Product isolation by combining chromatography on AG1-X8 followed by adsorption/desorption on/from charcoal proved to be the most useful compromise. A final gel filtration on Sephadex LH-20 afforded a product that was free of formate[46] but still slightly contaminated with 7 (10%, w/w), a byproduct of the enzymatic reaction.[47] Compound 5 could be obtained in 10-mmol-scale experiments based on GlcNAc-6-P in 17% yield (~90% purity).

Chemical Synthesis. The "phosphomorpholidate" procedure, which involves the coupling of suitable salt of a sugar-α-1-phosphate with a nucleoside phosphomorpholidate, has been widely used for the synthesis of various sugar nucleotides.[19,48] Compounds 5 and 7 have been prepared by this route in about 60% yield.[19] Our goal was to design a practical procedure to generate the α-1-phosphoryl anomer of GlcNAc on large (millimole) scale.

We first examined the MacDonald reaction.[49] In this procedure the anomeric acetoxy group of a peracetylated sugar is displaced by phosphoric acid to give a mixture of α and β anomers of the phosphate. We applied this reaction to the conversion of pentaacetylglucosamine 8 to its phosphate analogue 9 (eq 1). The overall yield of 9
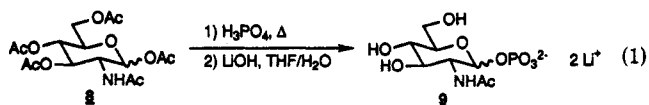
was low (20–50%) and the α/β ratio varied from 3/7 to 1/1. Attempts to obtain the pure α-anomer by recrys-

tallization were unsuccessful. We therefore investigated a stereospecific route to the α-anomer and selected a sequence which could be scaled up conveniently (Scheme III). The α-chlorotetraacetylglucosamine 10 was easily obtained by chloroacetylation of GlcNAc.[50] The intramolecular cyclization to the oxazolidino sugar[51] proceeded in almost quantitative yield. Because it proved difficult to eliminate the impurities present in large-scale preparations of 10, we developed a cleaner route to the oxazolidinoglucosamine 11. Activation of pentaacetylglucosamine 8 with TMS triflate proceeded in high yield and was amenable to large-scale preparation of 11.

The phosphorylation of 11 with dibenzyl phosphate[52-54] afforded the α-anomer exclusively, although both anomers are reported to form at the beginning of the reaction.[53] The catalytic hydrogenolysis of the benzyl phosphate 12 was conducted in the presence of a stoichiometric amount of cyclohexylamine to serve as a buffer to prevent the acid-catalyzed degradation of the product 14. The cyclohexylammonium salt of 14 was normally of high purity, but could be further purified by recrystallization.

The last step in the sequence used the Khorana–Moffatt ("phosphomorpholidate") procedure[48] to couple a morpholidate derivative of UMP and 14. We modified the classical conditions slightly: DMF was used as solvent instead of pyridine to reduce problems of solubility, and molecular sieves were used to ensure dryness. The coupling reaction required 5–6 days to proceed to completion, but the product was very clean as judged from ^{31}P NMR spectroscopy. These results are in agreement with an earlier report (5 days, 35%),[55] but the yield was lower than that reported in the original work (5 days, 60–70%).[48]

Conclusion

The enzymatic route to UDP-GlcNAc 5 using cell ex-

(46) Lyophilization to remove the formate resulted in decomposition of UDP-GlcNAc even if the solution was buffered at pH 7 with triethylamine.

(47) The α-H1 of UDP-GlcN was shifted slightly downfield (5.58 ppm) relative to the α-H1 of UDP-GlcNAc (5.48 ppm). Control experiments assured that the hydrolysis was not chemical. It remained unclear whether GlcNAc-6-P, GlcNAc-1-P, or UDP-GlcNAc was hydrolyzed enzymatically.

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tracts from the yeast *C. utilis* (Scheme II, route B) for the coupling reaction between GlcNAc-6-P (**3**) and UTP is a useful alternative to the chemical synthesis. Although the yield of the condensation step was lower than in the chemical approach to UDP-GlcNAc (17% from GlcNAc-6-P and 38% from GlcNAc-1-P, respectively), its practicability is provided by the ready availability of the starting materials: UTP can be obtained in a coupled reaction sequence from CMP in >100-mmol scale,[16] and the experimental procedures in this paper provide a convenient access to GlcN-6-P (**2**) and its *N*-acetyl derivative **3** on a gram scale. In comparison to the recently reported chemoenzymatic route for UDP-GlcNAc employing fractionated enzyme extracts from calf liver for the condensation of chemically prepared GlcNAc-1-P and UTP,[29] the enzyme-based methods developed in this investigation are more convenient. The yields obtained with calf liver extracts were reported to be higher, but its practicability has so far only been reported on submillimole scale. An efficient method for preparing these enzymes might make this procedure competitive.

The chemical synthesis we used (Scheme III) required five steps, with an overall yield of 15%. The key reaction is the selective formation of the tetraacetylglucosamine-α-1-phosphate **13**, which could be obtained in high yields. The major drawback in this synthesis is also the modest yield of the final coupling reaction between GlcNAc-α-1-P and UMP-morpholidate (38% from GlcNAc-1-P). In contrast to the enzymatic strategy, the chemical route provides access to structural analogs of UDP-GlcNAc.

Both the chemical and enzyme-based approaches investigated in this paper are applicable for the gram-scale preparation of UDP-GlcNAc. The overall yields of UDP-GlcNAc obtained in the different routes were comparable. The chemoenzymatic procedures are more convenient in practice than those of the chemical synthesis; the purification of product from the complex reaction mixture is, however, more laborious.

Experimental Section

General Methods and Materials. Reagents and solvents were reagent grade and used as received unless otherwise noted. THF was distilled from sodium benzoquinone ketyl before use, CH_2Cl_2 from CaH_2. DMF, MeCN, and dichloroethane were Spectrograde or Gold Label grade and stored over activated 4-Å molecular sieves under argon. TLC were performed on glass plates with UV fluorescent indicator (Merck, silica gel 60 F254) and were stained with a mixture of *p*-anisaldehyde/acetic acid/sulfuric acid/ethanol (5.5/2.2/7.5/200, v/v/v/v) or with the Dittmer–Lester reagent for compounds containing phosphorus.[56] Preparative TLC was performed on 20-20 silica plates (Merck, Silica gel 60 F254). Flash chromatography[57] employed 40–63-μm silica (Merck, silica gel). Ion-exchange resins AG1-X8 and AG1-X2 (formate form, 100–200 mesh) and Dowex 50W-X8 (H⁺ form, 100–200 mesh) were purchased from Biorad. Sephadex LH-20 was used as received from Pharmacia.

^1H NMR spectra were obtained at 300, 400, and 500 MHz, ^{13}C at 75.45 and 100 MHz, and ^{31}P at 121.49 MHz. ^1H chemical shifts in D_2O are referenced to HOD set at 4.8 ppm and in $CDCl_3$ to TMS set at 0.0 ppm. ^{31}P shifts are relative to external 85% H_3PO_4 set at 0.0 ppm; ^{13}C shifts are relative to external dioxane set at δ 67.6 ppm.

All enzymes were obtained from Sigma: hexokinase (EC 2.7.1.1, from bakers' yeast, Sigma H 5375), pyruvate kinase (EC 2.7.1.40, from rabbit muscle, Sigma P 9136), acetate kinase (EC 2.7.2.1, from *E. coli*, Sigma P 7779), phosphoglucomutase (EC 5.4.2.2, from rabbit muscle, Sigma P 3397), UDP-Glc pyrophosphorylase (EC 2.7.7.9, from bovine liver, Sigma U 5877), and inorganic pyro-

phosphatase (EC 3.6.1.1, Sigma I 4503). Dried yeast cells of *C. utilis* (Torula yeast) were also received from Sigma (YCU).

Assays. Hexokinase was assayed by the procedure of Scheer et al.[58] or more conveniently by the procedure described by Bergmeyer.[59] The other enzymes used were assayed according to standard procedures.[60] Protein concentration was determined spectrophotometrically according to the method described by Bradford[61] referencing the calibration curve to bovine serum albumin as the standard protein.

Enzymatic Route. Glucosamine-6-phosphate (2). To 100 mL of water were added successively GlcN·HCl (2.16 g, 10 mmol, 1 equiv), ATP·Na_2 (100 mg, 0.17 mmol, 0.017 equiv), and $MgCl_2$ (83 mg, 0.4 mmol, 0.04 equiv). After each addition, the pH of the solution was adjusted to 7.5 using 2.5 M NaOH. Nitrogen was then bubbled through the solution for 20 min. Acetyl phosphate (lithium potassium salt, 2.4 g, 12 mmol, 1.2 equiv) was added, followed by acetate kinase (90 units) and hexokinase (1660 units). The solution was stirred at 26 °C under nitrogen and monitored by TLC (eluent: *n*-PrOH/NH_4OH/H_2O, 7/3/2, v/v/v) and ^{31}P NMR. After 5 h, the reaction was quenched by addition of $BaCl_2$ (3.66 g, 15 mmol, 1.5 equiv). The white precipitate that formed (mainly barium phosphate) was removed by filtration and rinsed with water. To the filtrate was added ethanol (150 mL); the resulting cloudy solution was allowed to crystallize at 4 °C for 2 d. After filtration and drying in vacuo, the barium salt was obtained as a white solid[62] (3.51 g, 88% yield). A sample acidified by mixing with an ion-exchange resin (Dowex 50W-X8, H⁺ form) and neutralized with 5 N NaOH was indistinguishable from authentic material (Sigma) by TLC, IR, and ^1H and ^{31}P NMR analyses.

This procedure has been scaled up to 20 g of 1 (93.6 mmol). In this case, acetyl phosphate was prepared by the reported procedure[63] and was obtained as a suspension. The phosphorylation procedure was slightly modified: in order to minimize hydrolysis, the acetyl phosphate suspension (150 mL, ~140 mmol) was kept at 0 °C and added in portions (3 × 50 mL) to the reaction mixture (containing $MgCl_2$, 1.2 g; ATP, 1.05 g; acetate kinase, 225 units; hexokinase, 8300 units in a volume of 800 mL). Equal quantities of acetate kinase and hexokinase were added after 3 h. The same workup as above yielded glucosamine-6-P (**2**) as the yellowish barium salt (24.86 g, 66%).

Study of the "Browning Reaction". A solution of GlcN·HCl (42 mg, 0.19 mmol) in D_2O (1 mL) was neutralized to pH 7.5 with a solution of KOD in D_2O. This mixture was divided in two equal parts: to one was added PEP (monosodium salt, 21 mg, 0.1 mmol, 1 equiv), to the other was added pyruvate (11 mg, 0.1 mmol, 1 equiv). The two solutions were adjusted to pH 7.5 and poured into two NMR tubes. The course of the reaction was monitored by ^1H NMR. The same procedure was repeated for **2**.

***N*-Acetoxysuccinimide (NAS).** To a suspension of *N*-hydroxysuccinimide (NHS, 4.17 g, 36.3 mmol, 1 equiv) in dry methylene chloride (100 mL) under nitrogen was added dry triethylamine (5.3 mL, 38.1 mmol, 1.05 equiv). After the resulting clear solution was cooled to 0 °C, acetyl chloride (2.7 mL, 38.1 mmol, 1.05 equiv) was slowly added. The mixture was stirred for 2 h at rt. Ether (100 mL) was added, and the resulting precipitate of Et_3N·HCl was removed by filtration. After evaporation of the solvent, NAS was obtained as a white solid (5.62 g, 95% pure as judged by ^1H NMR, 93% yield): ^1H NMR ($CDCl_3$) δ 2.81 (s, 4 H), 2.30 (s, 3 H); (D_2O) δ 2.95 (s, 4 H), 2.40 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 169.08, 165.42, 25.36, 17.25.

***N*-Acetylglucosamine-6-phosphate (3).** To a solution of glucosamine-6-P (**2**) (2.46 g, 9.4 mmol, 1 equiv) in water (15 mL) at pH 7.5 was added a solution of NAS (1.63 g, 10.4 mmol, 1.1 equiv) in THF/water (3 mL/9 mL). The pH was maintained at 7.5 by adding 2.5 N NaOH. The pH remained constant after 1

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(62) Upon drying, some samples turn slightly yellow or orange without any noticeable change in their NMR spectra.

(63) Crans, D. C.; Whitesides, G. M. *J. Org. Chem.* **1983**, *48*, 3130.

h. BaCl$_2$ (2.48 g, 12.3 mmol, 1.3 equiv) was added, and the resulting precipitate was removed by filtration. Acetone (~1 volume) was added, and the barium salt was allowed to crystallize at 4 °C for 12 h. After filtration and drying in vacuo, GlcNAc-6-P·Ba was obtained as a yellowish solid. A sample was acidified with ion-exchange resin (Dowex 50W-X8, H$^+$ form), was neutralized with 5 N NaOH, and was compared to the authentic compound (Sigma) by TLC and ^{1}H and ^{13}C NMR analyses (5.13 g, ~75% purity, 80% yield).[64]

A one-pot procedure has also been used: to a reaction mixture from a hexokinase-catalyzed phosphorylation of 1 (from 1 mmol of GlcN·HCl) was added a few drops of concentrated HCl to denature the enzymes. The pH was adjusted to 7.8, and NAS (235 mg, 1.5 mmol) was added as a solid. After this step, the same procedure as above was applied. An ~90% yield of 3 was estimated from the ^{1}H NMR spectrum of the white solid thus obtained.

Preparation of UDP-GlcNAc 5 with Crude Enzyme Extracts from *C. utilis*. Dried cells of the yeast *C. utilis* (10 g) were suspended in 80 mL of Tris/HCl buffer (50 mM, pH 7.5) containing 2-mercaptoethanol (5 mM) and stirred at 4 °C for 2 h to destroy the cell pellets. The slurry was divided equally among four 50-mL disposable centrifuge tubes, and glass beads[65] (~12 g) were added to each tube. The cells were disrupted by vigorous mixing of the sealed tubes on a vortex laboratory mixer for about 8 min with intermediate periods of cooling on ice. The supernatant liquid was decanted, the glass beads in each tube were washed with 10 mL of the same buffer, and the wash was combined with the supernatant. The cell fragments were sedimented by centrifugation (8000g, 4 °C, 10 min) and discarded. To the supernatants was added protamine sulfate (120 mg dissolved in 2 mL water at 50 °C), and the reaction mixture was stirred at 4 °C for 15 min. The resulting cloudy precipitate was removed by centrifugation (15000g, 4 °C, 15 min), and the supernatant (~115 mL) was dialyzed at 4 °C overnight against 3.5 L of Tris/HCl buffer (10 mM, pH 7.5) containing 2-mercaptoethanol (5 mM). The protein concentration of the dialyzed extracts was determined at ~13 mg/mL. This protein solution was used for the following enzymatic reactions.

A suspension of N-acetylglucosamine-6-phosphate (3) (barium salt, ~75%, 5.8 g, 10 mmol) in 40 mL of water was stirred with ~30 mL of ion-exchange resin (Dowex 50W-X8, H$^+$ form) for 20 min. The resin was removed by filtration and washed three times with 20-mL portions of water. The combined clear filtrates were neutralized with 5 N KOH, divided equally in four portions (2.5 mmol in ~25 mL each), and used in the subsequent reaction step with the crude enzyme extracts.

To 500 mL of Tris/HCl buffer (10 mM, pH 7.5) containing MgCl$_2$·6H$_2$O (500 mg, 2.5 mmol, 0.25 equiv) and 2-mercaptoethanol (195 mg, 2.5 mmol, 0.25 equiv) in a 1-L Erlenmeyer flask were added Glc-1,6-di-P·K$_2$·H$_2$O (5 mg, 0.015 mmol, 0.0015 equiv), UTP·Na$_3$·2H$_2$O (~90%, 1.95 g, 3.0 mmol, prepared according to the method of Simon et al.[16]), a quarter of the GlcNAc-6-P solution, PPase (500 units), and the cell extracts obtained from *C. utilis*. The air in the flask was replaced by argon, and the flask was sealed with a gas-tight cap. The reaction was performed at rt and monitored by TLC (eluant: 1-propanol/ammonia/water, 6/3/2, v/v/v). As the starting materials disappeared, three further portions of the solution of GlcNAc-6-P (~25 mL, containing ~2.5 mmol, total amount 1 equiv) and UTP·Na$_3$·H$_2$O (~90%, 1.95 g, containing 3.0 mmol, total amount 1.2 equiv) were added at 6, 16, and 28 h, respectively. The reaction was stopped after 48 h by placing the reaction flask in a bath of boiling water for 3 min. The resulting precipitate was removed by filtration through Celite 545, and the filtrate was applied to the top of a column of ion-exchange resin (AG1-X8, formate form, 6 cm × 20 cm). The column was subsequently washed with 800 mL of water and 800 mL of 4 N formic acid. These washings were discarded. UDP-GlcNAc was eluted with 800 mL of 4 N formic acid containing

0.4 M ammonium formate. Activated charcoal (~100 g) was added to the fraction containing the UDP-GlcNAc and stirred for 30 min. The charcoal was recovered by filtration and washed with water (1.5 L). Compound 5 was desorbed from the charcoal by rinsing with 600 mL of a mixture of water/ethanol/ammonia (50/45/5, v/v/v). After concentration of the resulting solution of 5 in vacuo to ~40 mL, ~25 mL of ion-exchange resin (Dowex 50W-X8, H$^+$ form) was added and the suspension was stirred for 30 min. The resin was removed by filtration, and the filtrate was neutralized with 5 N NaOH. The solution was then concentrated in vacuo to a volume of ~4 mL and applied to a gel filtration column (Sephadex LH-20, 1 cm × 90 cm) and eluted with water. The collected fractions containing UDP-GlcNAc were concentrated to dryness in vacuo. The resulting residue gave 1.11 g of a crystalline, white UDP-GlcNAc (disodium salt, 90% purity, 17% yield), which was indistinguishable from authentic material (Sigma) by ^{1}H and ^{13}C NMR spectroscopy: ^{1}H NMR (400 MHz, D$_2$O) δ 7.92 (d, H6″, J = 8.1 Hz), 5.93 (d, H1′, J = 4.9 Hz), 5.91 (d, H5″, J = 8.1 Hz), 5.48 (dd, H1, J = 7.1, 3.4 Hz), 4.31 (m, H2′, H3′), 4.23 (m, H4′), 4.19 (m, H5′a), 4.12 (m, H5′b), 3.93 (dt, H2, J = 10.5, 2.9 Hz), 3.87 (ddd, H5, J = 10.1, 4.0, 2.2 Hz), 3.82 (dd, H6a, J = 12.4, 2.2 Hz), 3.79–3.72 (m, H6b, H3), 3.50 (t, H4, J = 9.6 Hz), 2.03 (s, Ac); ^{13}C NMR (100 MHz, D$_2$O) δ 175.6 (C=O, Ac), 167.3 (C4″), 152.7 (C2″), 142.5 (C6″), 103.5 (C5″), 95.3 (C1), 89.3 (C1′), 84.1 (d, C4′, J = 8.9 Hz), 74.6 (C3′), 73.9 (C5), 71.8 (C2′), 70.5, 70.3 (C3, C4), 65.8 (C5′), 61.2 (C6), 54.5 (d, C2, J = 8.3 Hz), 22.9 (CH$_3$, Ac).

Chemical Route. 2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucose (8). A suspension of GlcN·HCl (7.5 g, 34.9 mmol, 1 equiv) in a mixture of dry pyridine (50 mL) and acetyl chloride (50 mL, 53.0 mmol, 15 equiv) was stirred overnight at rt under nitrogen. The resulting clear solution was poured into a mixture of ice and water (200 mL) and extracted three times with chloroform (3 × 50 mL). The organic layer was washed twice with 50 mL of 1 N HCl, and then with water (50 mL), and finally with 75 mL of a saturated solution of CuSO$_4$. Drying over MgSO$_4$, filtration, and evaporation of the solvent produced a white solid (7.8 g, 58% yield): ^{1}H NMR (300 MHz, CDCl$_3$) δ 6.13 (d, H1, J = 3.8 Hz), 5.68 (br d, NH, J = 9.4 Hz), 5.25–5.10 (m, H3, H4), 4.45 (ddd, H2, J = 9.4, 9.3, 3.8 Hz), 4.21 (dd, J = 12.5, 4.1 Hz), 4.02 (dd, J = 12.5, 2.1 Hz), 3.98 (ddd, H5, J = 9.3, 4.1, 2.1 Hz), 2.15 (s, Ac), 2.05 (s, Ac), 2.01 (s, Ac), 2.00 (s, Ac), 1.89 (s, Ac); ^{13}C NMR (75.45 MHz, CDCl$_3$) δ 171.43, 170.49, 169.90, 168.98, 168.50 (C=O, 5 Ac), 90.75 (C1), 70.78, 69.83, 67.84 (C3, C4, C5), 61.75 (C6), 51.21 (C2), 23.05, 20.97, 20.76, 20.64 (CH$_3$, 5 Ac).

2-Acetamido-2-deoxy-α,β-D-glucose Dilithium 1-Phosphate (9). The reported procedure[12] to produce 9 was run on a scale of 4 g (10.2 mmol of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucose (8)), but the workup was modified. After 1 h of heating, dry THF was added when the black mixture was still hot (~50 °C, *CAUTION*: THF bp = 55 °C). It was difficult to dissolve the resulting black tar, and the yield was lower following the original procedure. After cleavage of the acetates and removal of Li$_3$PO$_4$ the brown solution was clarified by filtration through activated charcoal. Evaporation of the solvent gave a yellow solid that was recrystallized from a mixture of ethanol and water. The yield varied from 20% to 45%. The modification of Jeanloz et al.[66] was also used successfully to cleave the acetate groups: ^{1}H NMR (D$_2$O) δ 5.38 (dd, H1α, J = 7.5, 3.0 Hz), 4.95 (dd, H1β, J = 7.5, 7.5 Hz), 4.0–3.9 (m, 4 H), 3.85–3.7 (m, 4 H), 3.7–3.35 (m, 4 H), 2.07 (s, 3 H), 2.05 (s, 3 H).

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucosyl Chloride (10). A suspension of GlcN·HCl (10 g, 48.5 mmol, 1 equiv) in acetyl chloride (20 mL, 280 mmol, 5.77 equiv) was stirred overnight at rt under nitrogen. The resulting orange-pink clear solution was diluted with chloroform (80 mL) and poured onto ice (200 g). After mixing and decantation, the organic layer was washed with a mixture of ice and saturated sodium bicarbonate solution (100 mL). Drying over MgSO$_4$, filtration, and évaporation of the solvent produced a yellowish solid. Recrystallization from chloroform–ether gave a white solid (11.9 g) comprising the title compound (10) contaminated by the peracetylated glucosamine

(64) An impurity present at the level of ~25% was assigned by ^{1}H NMR as NHS (s, 2.67 ppm). The product was quantitatively acetylated as judged by ^{1}H NMR from comparison with authentic material (Sigma).

(65) The beads were washed several times with 6 N HCl and subsequently with an excess of water. Their diameter was between 0.25 and 0.30 mm.

(66) Warren, C. D.; Konami, Y.; Jeanloz, R. W. *Carbohydr. Res.* **1973**, *30*, 257.

α-GlcNAc$_5$. After flash chromatography (silica, eluant: ether/methanol, 95/5 to 80/20), the title product was isolated as 11.2 g (63% yield) of translucent prisms (it crystallized in the effluents during chromatography): ^{1}H NMR (500 MHz, CDCl$_3$) δ 6.17 (d, H1, J = 3.75 Hz), 5.91 (br d, NH, J = 8.75 Hz), 5.31 (dd, H3, J = 10.2, 10.2 Hz), 5.19 (dd, H4, J = 10.2, 10.2 Hz), 4.52 (ddd, H2, J = 10.2, 8.75, 3.75 Hz), 4.30–4.21 (m, H5 and H6 or H7), 4.11 (br d, H6 or H7, J = 11.2 Hz), 2.09 (s, Ac), 2.04 (2 s, 2Ac), 1.97 (s, Ac).

2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-gluco-pyrano)[2,1-*d*]-Δ²-oxazoline (11). From 10. To a suspension of anhydrous sodium bicarbonate (4.23 g, 50.4 mmol, 1.7 equiv) and tetraethylammonium chloride (4.23 g, 23 mmol, 0.8 equiv) in dry acetonitrile (35 mL) under nitrogen was added 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucosyl chloride (10) (10.57 g, 29 mmol, 1 equiv). The mixture was heated at 55 °C for 1 h and then evaporated to dryness. The residue was taken up in methylene chloride (250 mL). The resulting solution was extracted three times with water (100 mL), and each aqueous extract was extracted in turn with methylene chloride (100 mL). The organic layers were combined and dried over sodium sulfate. Evaporation of the solvent and flash chromatography of the residue (silica, eluent: ether/methanol, 98/2 to 95/5) gave the title product as a pale yellow gum (7.09 g, 74%).

From 8. To cloudy solution of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucose (8) (15 g, 41.5 mmol, 1 equiv) in dichloroethane (200 mL) containing activated molecular sieves under nitrogen was added dropwise a solution of trimethylsilyl triflate (9.3 mL, 65.5 mmol, 1.7 equiv). At the end of the addition, the solution became clear and slightly orange. TLC analysis revealed the formation of a compound, which is not the oxazolidinosugar, more polar than the starting material. After the reaction was stirred for 50 min, triethylamine (9.15 mL, 65.5 mmol, 1.7 equiv) was added. TLC analysis showed that the polar product disappeared to give the expected product. After 20 min, the mixture was filtered, concentrated in vacuo, and chromatographed (silica, eluent: ether/methanol, 98/2 to 95/5). The title product was obtained as a pale yellow gum (12.4 g, 91%): ^{1}H NMR (300 MHz, CDCl$_3$) δ 5.90 (d, H1, J = 7.5 Hz), 5.16 (dd, H3, J = 2.5, 2.5 Hz), 4.84 (ddd, H4, J = 9.0, 2.5, 1.0 Hz), 4.09 (d, H6, H7, J = 4.5 Hz), 4.05 (dddq, H2, J = 7.5, 2.5, 1.0, 1.1 Hz), 3.52 (dt, H5, J = 9.0, 4.5 Hz), 2.03 (s, Ac), 2.015 (s, Ac), 2.01 (d, CH$_3$, J = 1.1 Hz), 1.99 (s, Ac); ^{13}C NMR (75.45 MHz, CDCl$_3$) δ 170.58, 169.55, 169.23 (C=O, 3 Ac), 166.92, 99.52 (C1), 70.40, 68.46, 67.66, 64.88, 63.40 (C2, C3, C4, C5, C6), 20.84, 20.76, 20.68 (CH$_3$, 3 Ac), 13.87 (CH$_3$); IR (neat) 1740, 1670, 1365, 1030.

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucosyl Dibenzyl Phosphate (12). To a solution of 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)[2,1-*d*]-Δ²-oxazoline (11) (4.11 g, 12.5 mmol, 1 equiv) in dry dichloroethane (80 mL) under nitrogen was added a solution of dibenzyl phosphate (4.66 g, 16.8 mmol, 1.1 equiv) in dry dichloroethane (20 mL). Activated molecular sieves can be added to ensure dryness. The solution was kept under nitrogen at rt for 24 h. The solution was concentrated, and the residue was subjected to flash chromatography (silica, eluent: ether/methanol, 98/2 to 95/5). The title product was obtained as a colorless gum that crystallized upon standing at 0 °C (1.91 g, 61%).

The same procedure was repeated on a scale of ~10 g of 11 (10.27 g, 31.2 mmol) in 160 mL of dichloroethane and dibenzyl phosphate (11.66 g, 42 mmol in 40 mL of dichloroethane), but the yield was lower (49%): ^{1}H NMR (500 MHz, CDCl$_3$) δ 7.40–7.30 (m, 10 H), 6.01 (br d, NH, J = 9.14 Hz), 5.61 (dd, H1, J = 6.1, 3.3 Hz), 5.14 (dd, H3, J = 9.9, 9.6 Hz), 5.09 (dd, H4, J = 9.8, 9.9 Hz), 5.06 and 5.03 (AB, CH$_2$Bn, J = 8.9 Hz), 5.02 and 5.01 (AB, CH$_2$Bn, J = 8.7 Hz), 4.34 (dddd, H2, J = 9.1, 9.6, 3.32, 3.1 Hz), 4.09 (dd, H6 or H7, J = 12.5, 4.0 Hz), 4.01 (ddd, H5, J = 9.8, 4.0, 2.1 Hz), 3.89 (dd, H6 or H7, J = 12.5, 2.1 Hz), 2.01 (s, Ac), 1.98 (s, 2Ac), 1.70 (s, NHAc); ^{13}C NMR (75.45 MHz, CDCl$_3$) δ 171.28, 170.60, 169.29 (C=O, 3 Ac), 165.46 (C=O, NHAc), 129.09, 128.95, 128.50, 128.21 (Ph), 96.45 (d, C1, J = 7.5 Hz), 70.72 (CH$_2$Bn), 69.79, 67.59, 61.45 (C3, C4, C5, C6), 51.88 (d, C2, J = 8.07 Hz), 22.77 (NHAc), 20.90, 20.72, 20.62 (CH$_3$, 3 Ac).

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucosyl Dicyclohexylammonium Phosphate (13). To a degassed solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucosyl dibenzyl phosphate (12) (4.42 g, 7.25 mmol, 1 equiv) in a mixture of methylene chloride (35 mL) and methanol (15 mL) under nitrogen was added successively palladium on charcoal (1.12 g) and cyclohexylamine (1.90 mL, 16.5 mmol, 2.2 equiv). Hydrogen was bubbled into the solution for 15 min, and then positive hydrogen pressure was maintained in the flask. When the benzyl phosphate could no longer be detected by TLC (-1.5 h), the mixture was filtered and the solvents were evaporated, leaving a white solid. Recrystallization from chloroform–ether separated the excess ammonium salts. Evaporation of the filtrate gave the title product as a white foam (3.91 g, 86%): ^{1}H NMR (500 MHz, CDCl$_3$) δ 7.0–6.9 (br s NH), 5.54 (dd, H1, J = 7.0, 3.0 Hz), 5.38 (dd, H3, J = 10.0, 10.0 Hz), 5.27 (dd, H4, J = 10.0, 10.0 Hz), 4.43 (ddd, H2, J = 10.2, 10.0, 3.0 Hz), 4.27–4.21 (m, H5, H6), 4.17 (br d, H7, J = 11.2 Hz), 3.05 (br t, 2 H, J = 7.7 Hz), 2.16 (s, 3 H Ac), 2.12 (br d, 4 H, J = 10.6 Hz), 2.09 (s, 6 H, Ac), 2.00 (s, NHAc), 1.87 (br d, 4 H, J = 12.3 Hz), 1.72 (br d, 4 H, J = 10.6 Hz), 1.53–1.25 (m, 10 H).

2-Acetamido-2-deoxy-α-D-glucosyl Dicyclohexylammonium Phosphate (14). To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucosyl dicyclohexylammonium phosphate (13) (3.5 g, 5.5 mmol, 1 equiv) in dry methanol (25 mL) was added sodium methylate (980 mg, 18.5 mmol, 3.3 equiv). After 1 h at rt, ion-exchange resin (Dowex 50W-X8, H$^+$ form) was added to adjust the pH to 6.5–7.0. After filtration and evaporation, a yellowish solid was obtained (2.62 g). This solid was directly used in the coupling reaction.

2-Acetamido-2-deoxy-α-D-glucosyl Uridine Diphosphate (UDP-GlcNAc, 5). The solid prepared above 2-acetamido-2-deoxy-α-D-glucosyl dicyclohexylammonium phosphate (14) (~5.5 mmol) was dissolved in dry DMF (25 mL) and uridine 5′-monophosphomorpholidate (2.27 g, 3.32 mmol, 0.6 equiv, Sigma) was added. After 5 days at rt, the solution was concentrated and the residue was subjected to ion-exchange chromatography (AG1-X2, formate form). The column was washed with an excess of water; compound 5 was eluted by an increasing concentration of ammonium bicarbonate. Evaporation of the solvent yielded the title compound as the white ammonium salt (597 mg, 38% yield) indistinguishable by TLC and NMR spectroscopy from authentic material (Sigma).