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Chemoenzymatic synthesis of UDP-*N*-acetyl-α-D-galactosamine

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Abstract

A novel chemoenzymatic synthesis of UDP-*N*-acetyl-α-D-galactosamine starting from uridine 5′-monophosphate (UMP) and sucrose is reported. In an enzymatic repetitive batch mode UDP-glucose was generated in situ from UMP and sucrose by the combination of nucleoside monophosphate kinase (EC 2.7.7.4) and sucrose synthase (EC 2.4.1.13). The transfer of UMP from UDP-glucose by galactose-1-phosphate uridyltransferase (EC 2.7.7.12) yielded UDP-α-D-galactosamine. The equilibrium of the synthesis was forced to the product side by the addition of phosphoglucomutase (EC 2.7.5.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) were used to regenerate UTP and the cofactor NAD. The yield for the enzymatic step was 42%. Finally, UDP-α-D-galactosamine was acetylated chemically with *N*-acetoxysuccinimide. Product isolation was accomplished by anion-exchange chromatography and gel filtration. The overall yield was 34% and 82 mg UDP-*N*-acetyl-α-D-galactosamine were isolated.

Keywords: Nucleotide sugar; Sucrose synthase; Cofactor regeneration; UDP-*N*-acetyl-α-D-galactosamine

1. Introduction

The progress of carbohydrate biology during the last years proves the importance of glycoconjugate structures for molecular biology and pharmacology [1,2]. The research activities in this field have caused a growing interest in the synthesis of well-defined carbohydrate structures. Glycosyltransferases of the Leloir pathway are very selective catalysts with high substrate specificities and an absolute stereo- and regioselectivity for the glycosidic linkage. The practical use of the Leloir pathway in oligosaccharide synthesis depends on the availability of the glycosyltransferases as well as the nucleoside diphosphate sugars as donor substrates.

N-acetylgalactosamine (GalNAc) is an important monomer in the oligosaccharide chains of N- and O-glycans [3,4]. Some of the corresponding GalNAc glycosyltransferases have been cloned and characterized to build up O-glycan core structures [5]. Several syntheses of the donor substrate UDP-*N*-acetyl-α-D-galactosamine (UDP-GalNAc) have been reported yet [6–10]. They either used the

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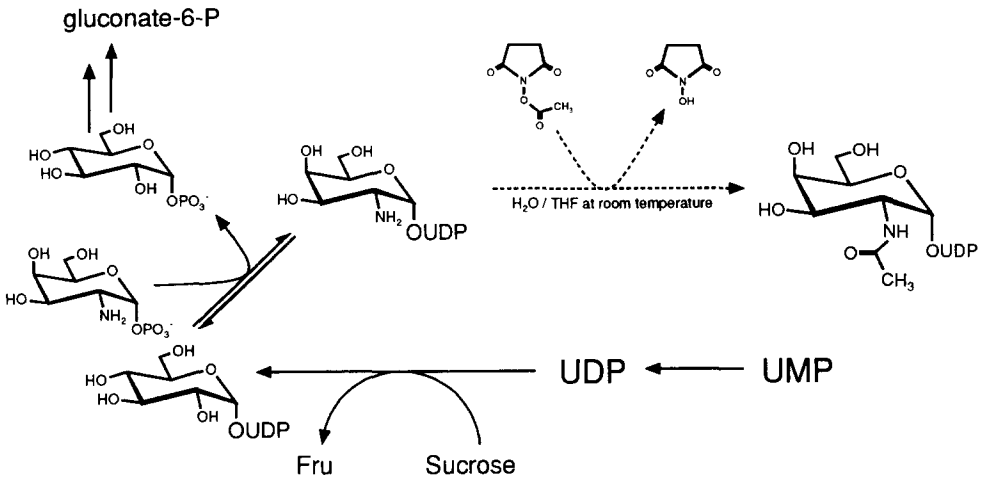

Scheme 1. General scheme for the chemoenzymatic synthesis of UDP-GalNAc starting from sucrose and uridine-5′ monophosphate (UMP).

chemical morpholidate-approach [7,8], were performed in a small scale [10] or involved rather complex compounds as substrates, like UTP [9] or UDP-glucose (UDP-Glc) [6].

In the present paper we report on the chemoenzymatic synthesis of UDP-GalNAc starting from sucrose and uridine-5′-monophosphate (Scheme 1). This was accomplished by the utilization of the plant glycosyltransferase sucrose synthase (SuSy) which cleaves sucrose with nucleoside diphosphates (NDP, N = U, dT, dU, A, C) yielding D-fructose and the corresponding activated glucoses [11]. In a combination of SuSy with kinases we synthesized UDP-, dUDP-, ADP- and CDP-glucose by the formation of the expensive NDP from unexpensive nucleoside monophosphate (NMP) [12]. The latter approach is now exploited in combination with further enzymes to realize the synthesis of UDP-$GalNH_2$ from relatively cheap compounds.

2. Results and discussion

The preparative synthesis of UDP-GalNAc from sucrose and uridine 5′-monophosphate (UMP) was divided in the enzymatic synthesis of UDP-galactosamine (UDP-$GalNH_2$) and the chemical acetylation of this intermediate, because there is no enzyme available for the acetylation of aminosugars (Fig. 1)

Development of the synthesis.—In the enzymatic synthesis of UDP-galactosamine (UDP-$GalNH_2$) we used SuSy from rice grains to catalyse the cleavage of sucrose with UDP to fructose and UDP-Glc. Therefore, in contrast to former syntheses there is no need to use stochiometric amounts of UDP-Glc or to regenerate this activated sugar. The commercially available galactose-1-phosphate uridyltransferase (URI) catalyses the transfer of UMP from UDP-Glc to galactosamine-1-phosphate

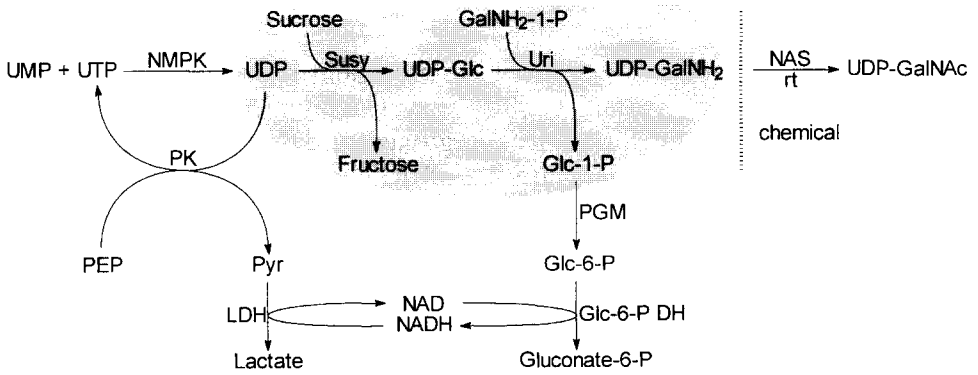

Fig. 1. Enzymatic and chemical part of the UDP-GalNAc synthesis. NMPK: Nucleoside monophosphate kinase; PK: pyruvate kinase; LDH: lactate dehydrogenase; Susy: sucrose synthase; Uri: galactose-1-phosphate uridyltransferase; PGM: phosphoglucomutase; G-6-P-DH: glucose-6-phosphate dehydrogenase; NAS: *N*-acetoxysuccinimide. The two-enzyme-reaction has a grey background.

($GalNH_2$-1-P) yielding UDP-$GalNH_2$. The two-enzyme-reaction (Fig. 1, grey background) yielded only 29% UDP-$GalNH_2$ and 23% of the intermediate UDP-Glc remained in the reaction mixture even when the substrate was used in excess (1.5 equivalents) (Fig. 2). The yield of UDP-$GalNH_2$ in this two-enzyme-reaction was limited by the unfavourable equilibrium constant (K_{eq} = 0.26) of the URI-catalysed reaction for the unnatural substrate galactosamine-1-phosphate. Therefore glucose-1-phosphate (Glc-1-P), the second product of this reaction, was removed from the equilibrium by two additional enzymatic reactions. Glc-1-P was isomerised to glucose-6-phosphate (Glc-6-P) by means of the enzyme phosphoglucomutase (PGM). Glucose-6-phosphate was oxidised to gluconate-6-phosphate by glucose-6-phosphate dehydrogenase (G-6-P-DH). The cofactor NAD of the latter reaction was regenerated in situ by the reaction catalysed by lactate dehydrogenase (LDH) (Fig. 1). In order to start the synthesis of UDP-$GalNH_2$ essentially from UMP, we synthesized UDP in situ from UMP and UTP catalysed by nucleoside monophosphate kinase (NMPK). To avoid the addition of quantitative amounts of UTP this substrate was regenerated by pyruvate kinase (PK). This extended synthesis yielded 43% of the product UDP-$GalNH_2$ relative to the initial amount of $GalNH_2$-1-P with less than 0.1% of the intermediate UDP-Glc in the raw product mixture (Fig. 2). In conclusion, a complete shift of the reaction equilibrium was achieved by the seven-enzyme-reaction and it was used for the preparative synthesis of UDP-$GalNH_2$.

Preparative synthesis.—In order to avoid high enzyme costs and to achieve high enzyme productivities (mg product/U enzyme) we used all enzymes repetitively in a batch mode [13]. After each batch the product-containing solution was separated from the enzymes by ultrafiltration and the next batch was started by the addition of a fresh substrate solution. In this way seven batches yielded 42% UDP-$GalNH_2$ in 226 h (Fig. 3). In spite of the long reaction time the reduction of enzyme activity was only moderate due to the enzyme stabilising effect of a high sucrose concentration in the reaction solution. The stabilising effect of sucrose in context with the application of sucrose synthase has also been exploited in our previously published syntheses of activated glucose and *N*-acetyllactosamine [12,14].

A quantitative yield of UDP-GalNAc was achieved in the chemical acetylation using *N*-acetoxysuccinimide [9]. The resulting raw product solution contained UDP-GalNAc as the sole nucleotide sugar, because the two other activated sugars occurring throughout the synthesis, UDP-Glc and UDP-$GalNH_2$ were converted completely in the enzymatic and chemical step, respectively. This facilitated the product isolation, because especially the separation of UDP-Glc and UDP-GalNAc was difficult to achieve. Consequently, the yield of the product isolation was comparatively high, achieving 81%. The overall yield amounted to 34% and 82 mg (133 μmol) UDP-GalNAc were produced.

In conclusion, our results demonstrate, that the application of the enzymes SuSy and NMPK to produce UDP-Glc in situ from UMP and sucrose as well as the repetitive use of all enzymes is the key to an economic synthesis of UDP-GalNAc.

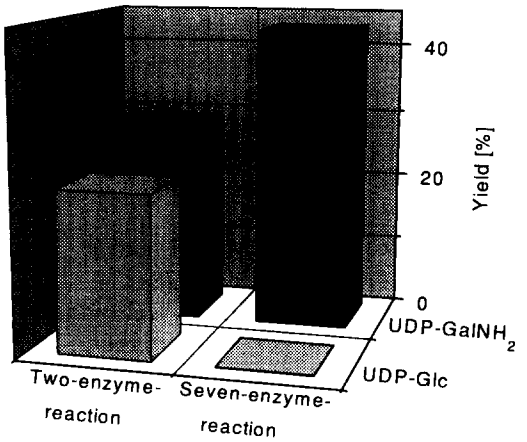

Fig. 2. Relative amounts of UDP-$GalNH_2$ and the intermediate UDP-Glc in the two and seven enzymes-reaction.

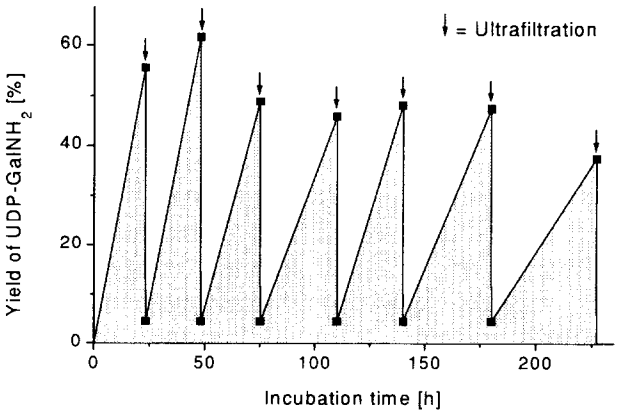

Fig. 3. Time-course of the repetitive batch synthesis. Seven batches were run in 226 h.

3. Experimental

General methods.—^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM-400 spectrometer. All reactions were followed by HPLC [11]. Nucleoside monophosphate kinase, pyruvate kinase, alkaline phosphatase, lactate dehydrogenase, phosphoglucomutase and glucose-6-phosphate dehydrogenase were from Boehringer (Mannheim, Germany). UMP (sodium salt), UTP (sodium salt), UDP-glucose (sodium salt), galactosamine-1-phosphate (sodium salt) and galactose-1-phosphate uridyltransferase from yeast were from Sigma (Deisenhofen, Germany). Sucrose synthase was prepared as previously described [15,16].

*Synthesis of UDP-*N*-acetyl-*α*-D-galactosamine.* —The enzymatic synthesis of UDP-GalNH$_2$ was performed using the repetitive batch technique. 20 mL of 200 mM Hepes-NaOH buffer adjusted to a pH of 7.7 containing 2.1 mM UMP (a), 2.8 mM GalNH$_2$-1-P (b), 0.03 mM NAD (c), 500 mM sucrose (d), 0.002 mM Glc-1,6-bisphosphate (e), 2 mM dithiothreitol (f), 2 mM $MgSO_4$ (g), 2.1 mM phospho(enol)pyruvate (PEP) (h), 0.2 mM UTP (i), 0.2 mM ATP (j), 10 mM K_2SO_4 (k), 20 mg BSA, 2 U SuSy, 40 U Uri, 8 U PGM, 8 U Glc-6-P-DH, 160 U LDH, 300 U PK and 10 U NMPK were stirred at an incubation temperature of 30 °C. After an incubation time of 20 to 40 h the conversion was complete. The reaction mixture was transferred into a stirred ultrafiltration cell (Amicon, model 8050, with a YM 30 membrane). The eluate was collected and stored at −20 °C. The 2 mL of retentate, containing the enzymes and BSA were used for the next batch. Substrates (a)–(k) were added in the corresponding volume of buffer to readjust the initial concentrations and volume. The resulting solution was then treated as the first batch. Seven batches yielding 120 mL of combined filtrates containing 164 μmol UDP-GalNH$_2$ were obtained. For the chemical acetylation [9] the pH of the product solution was adjusted to 7.5. 24 equivalents (613 mg) N-acetoxysuccinimide [17] in 1:1 THF–H_2O (7.5 mL) were added and after 10 h the conversion was complete. The product solution was incubated with 1 U/mL alkaline phosphatase for 15 h at 30 °C. This procedure converts the small amounts of UMP left in the product solution to uridine which is easier to be separated from UDP-GalNAc. After separation of the enzyme by ultrafiltration the filtrate was applied onto a column of anion-exchange resin (Dowex 1×2 in Cl^- form, 100–200 mesh, Ø 2.6 cm, length 26 cm) (Serva), which was equilibrated with 1 L of water. The column was washed with water until the conductivity of the eluate reached the value measured before the application of the sample. The product was eluated in a LiCl-gradient of 0–0.5 M at a LiCl-concentration of ~0.3 M. The fractions containing UDP-GalNAc were combined and concentrated at reduced pressure to a volume of 20 mL. The sample was desalted by passing through a gel filtration column (Sephadex G-10, Ø 2.6 cm, length 93 cm) (Pharmacia) using water as eluent. Lyophilisation yielded UDP-GalNAc (dilithium salt, 82 mg, 34%), indistinguishable from authentic material by ^{1}H and ^{13}C NMR spectroscopy: ^{1}H NMR (400 MHz, D_2O, Me_4Si): δ 7.95 (d,1 H, $^3J_{5'',6''}$ 8.1 Hz, H-6''), 5.99 (d, 1 H, $^3J_{1',2'}$ 4.7 Hz, H-1'), 5.97 (d, 1 H, H-5''), 5.56 (dd, 1 H, $^2J_{1,P}$ 7.1, $^3J_{1,2}$ 3.3 Hz, H-1), 4.41–4.32 (m, 2 H, H-2',3'), 4.32–4.15 (m, 5 H, H-4',5'a,5'b,5,2), 4.05 (d, 1 H, $^3J_{4,5}$ 2.5 Hz, H-4), 3.97 (dd, 1 H, $^3J_{2,3}$ 11.0, $^3J_{3,4}$ 3.1 Hz, H-3), 3.79 (dd, 1 H, $^3J_{5,6a}$ 7.0, $^2J_{6a,6b}$ 11.8 Hz, H-6a), 3.74 (dd, 1 H, $^3J_{5,6b}$ 5.1 Hz, H-6b), 2.09 (s, 3 H, Ac); ^{13}C NMR (100 MHz, D_2O, dioxane): δ 175.7 (C=O, Ac), 167.0 (C-4''), 152.6 (C-2''), 142.4 (C-6''), 103.4 (C-5''), 95.5 (d, $^2J_{1,P}$ 6.1 Hz, C-1), 89.2 (C-1'), 84.0 (d, $^3J_{4',P}$ 9.1 Hz, C-4'), 74.5 (C-3'), 72.8 (C-5), 70.4 (C-2'), 69.1 (C-4), 68.4 (C-3), 65.7 (d, $^2J_{5',P}$ 5.5 Hz, C-5'), 61.8 (C-6), 50.5 (d, $^3J_{2,P}$ 8.1 Hz, C-2), 22.9 (CH_3, Ac).

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