Pergamon

Tetrahedron, Vol. 52, No. 7, pp. 2395-2404, 1996
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0040-4020/96 $15.00 + 0.00

0040-4020(95)01081-5

Combined Enzymatic Synthesis of Nucleotide (Deoxy) Sugars from Sucrose and Nucleoside Monophosphates

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Abstract: The synthesis of NDP-glucose **3a-d** (N= A, C, U, dU) with sucrose synthase **B** was combined with the enzymatic synthesis of nucleoside diphosphates **2a-d** from their corresponding nucleoside monophosphates **1a-d** by different kinases **A**. Further combination with recombinant dTDP-glucose 4,6-dehydratase **D** enabled us to synthesize dUDP-6-deoxy-α-D-*xylo*-4-hexulose **5** from **1d** on a preparative scale. By using the repetitive batch technique the enzymatic syntheses of nucleotide (deoxy) sugars **3a-d**, **5** could be realized on a 0.1 - 0.5 g scale.

INTRODUCTION

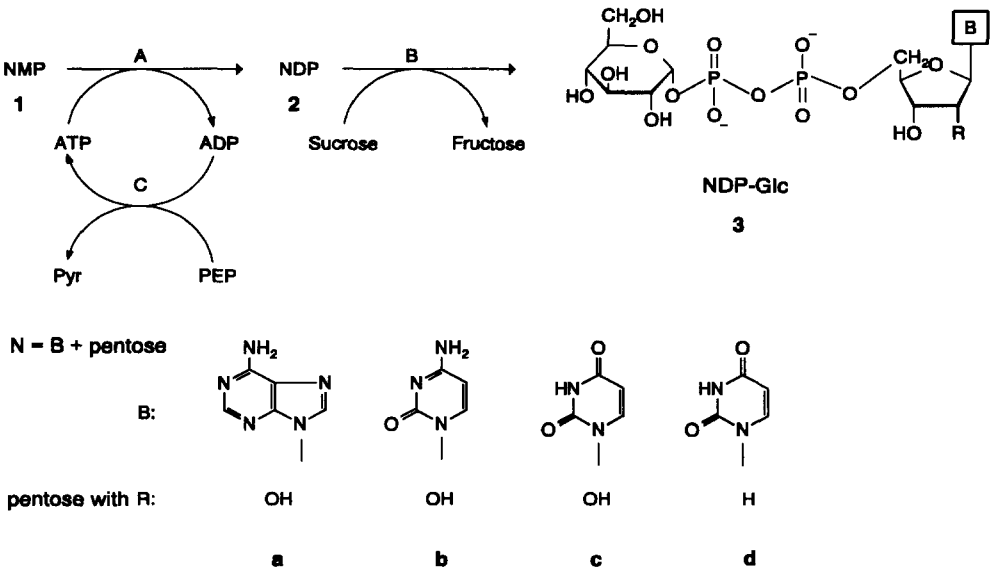
During the last decade it became evident that oligosaccharide residues of glycoconjugates are involved in important intra- and intermolecular communication events leading to an increased demand for glycoconjugates as research and therapeutic targets.[1] Since the chemical synthesis of oligosaccharides is quite difficult and tedious including the complex protection and deprotection chemistry of reactive groups as well as problems of stereocontrolled synthesis[2] glycosyltransferases and glycosidases have been applied because of their high regio- and stereoselectivity without the need for protection.[3,4] The utilization of glycosyltransferases requires nucleotide sugars. Apart from the chemical preparation of nucleotide sugars some large-scale, enzymatic syntheses have been developed by using highly specific pyrophosphorylases.[5,6] Other important nucleotide sugars such as ADP-Glc[7] **3a**, CDP-Glc[8] **3b** and dUDP-Glc[9] **3d** have been synthesized enzymatically only on a small scale.

Sucrose synthase (**B**, SuSy, EC 2.4.1.13) from rice grains catalyzes the cleavage of sucrose **4** with uridine-5'-(UDP) **2c**, 2'-deoxythymidine-5'-(dTDP), 2'-deoxyuridine-5'-(dUDP) **2d**, adenosine-5'-(ADP) **2a** and cytidine-5'-(CDP) diphosphate **2b** to their corresponding activated glucoses **3a-d** and D-fructose.[10,11]

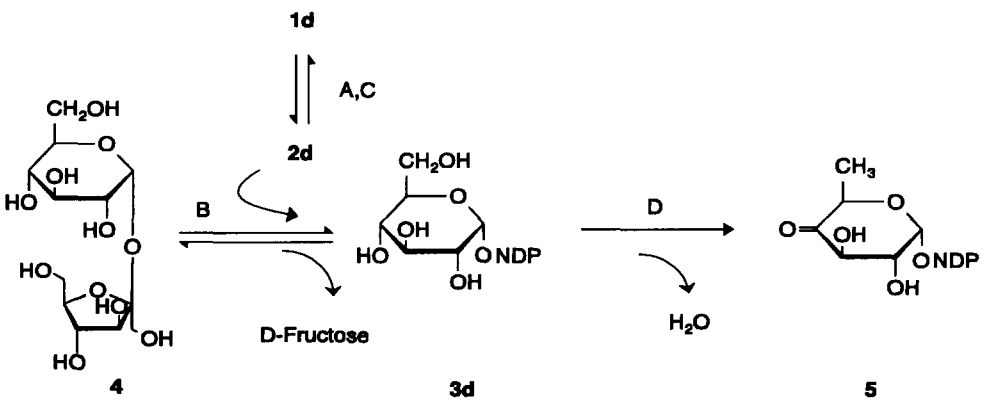
Nucleoside diphosphates (NDP) are still expensive substrates, which can be obtained from much more cheaper nucleoside monophosphates (NMP) by chemical or enzymatic synthesis. In the present paper we combined the SuSy catalyzed synthesis (**B**) of activated glucoses (ADP- **3a**, CDP- **3b**, UDP- **3c**, dUDP-Glc **3d**) with the enzymatic formation of NDP **2a-d** from NMP **1a-d** catalysed by nucleoside monophosphate kinase (**A**, NMPK, EC 2.7.4.4) or myokinase (**A**, MK, EC 2.7.4.3) including *in situ* regeneration of ATP with pyruvate kinase (**C**, PK, EC 2.7.1.40) (Scheme 1).

Further combination with dTDP-glucose 4,6-dehydratase (**D**, EC 4.2.1.46) yielded dUDP-6-deoxy-α-D-*xylo*-4-hexulose **5** in a one pot synthesis starting from **1d** and **4** (Scheme 2). The synthesis of this analogue of dTDP-6-deoxy-α-D-*xylo*-4-hexulose discloses now a convenient access to the key intermediate in biosynthetic pathways of many activated D- and L-deoxysugars, e.g. dTDP-L-mycarose, dTDP-L-rhamnose or dTDP-L-dihydrostreptose.[12,13] dUDP-6-deoxy-α-D-*xylo*-4-hexulose **5** may be also tested as substrate or inhibitor of

glycosyltransferases involved in these pathways. Recently, we utilized **5** for the reactivation of reductively inactivated UDP-glucose 4'-epimerase (EC 5.1.3.2) during the enzymatic syntheses of *N*-acetyllactosamine and analogues thereof..[14]



Scheme 1: Synthesis of activated glucoses from nucleoside monophosphates with nucleoside monophosphate kinase or myokinase **A**, sucrose synthase **B** and pyruvate kinase **C**. NMP nucleoside monophosphate; NDP nucleoside diphosphate; NDP-Glc nucleoside diphosphate glucose; ATP adenosine-5′-triphosphate; PEP phospho(enol)pyruvate; Pyr pyruvate..



Scheme 2: Synthesis of dUDP-6-deoxy-α-D-*xylo*-4-hexulose **5** with nucleoside monophosphate kinase **A**, pyruvate kinase **C**, sucrose synthase **B**, dTDP-glucose 4,6-dehydratase **D**; NDP nucleoside diphosphate.

RESULTS

In order to optimize the synthesis of activated (deoxy) sugars with sucrose synthase in combination with different kinases we tested the substrate spectrum of the kinases and different important parameters e.g. pH optimum and stability.

Substrate specifity of kinases

The substrate specifities of myokinases (MK) from rabbit, porcine and chicken muscle and nucleoside monophosphate kinase (NMPK) from bovine liver were tested in a photometric assay (Table 1).

Table 1: Substrate Specificities of Different Kinases

	Relative Activity* [%]			
NMP	MK porcine	MK chicken	MK rabbit	NMPK bovine
AMP (**1a**)	100	100	100	100
CMP (**1b**)	18	10	24	58
UMP (**1c**)	6	4	8	82
GMP	8	4	12	20
dUMP (**1d**)	4	2	8	18
dTMP	0	0	0	0

* the relative activity is based on the activity of the enzymes with AMP

MK from rabbit muscle was chosen for the synthesis of **3a** and **3b**. The enzyme was unstable in diluted solution but could be stabilised by the addition of 1 $mg*ml^{-1}$ BSA.[15] NMPK was used for the preparation of **3c,d** and **5**.

Determination of pH optima for the synthesis of nucleotide sugars

The synthesis of **3a** with MK, PK and SuSy as well as the synthesis of **3d** with NMPK, PK and SuSy was carried out at different pH values between 7.0 and 8.0. The pH optima for the synthesis of **3a,b** with MK and **3c,d** with NMPK was pH 7.5 and pH 7.2, respectively.

Preparative Synthesis of nucleotide (deoxy) sugars

The excellent stability of all enzymes enabled us to perform the synthesis of nucleotide (deoxy) sugars with the repetitive batch technique over several days. Figure 1 shows the course of the synthesis of **5** over four days. The enzymes were recovered from the product solution by an ultrafiltration step and directly reused by the addition of fresh substrate solution.

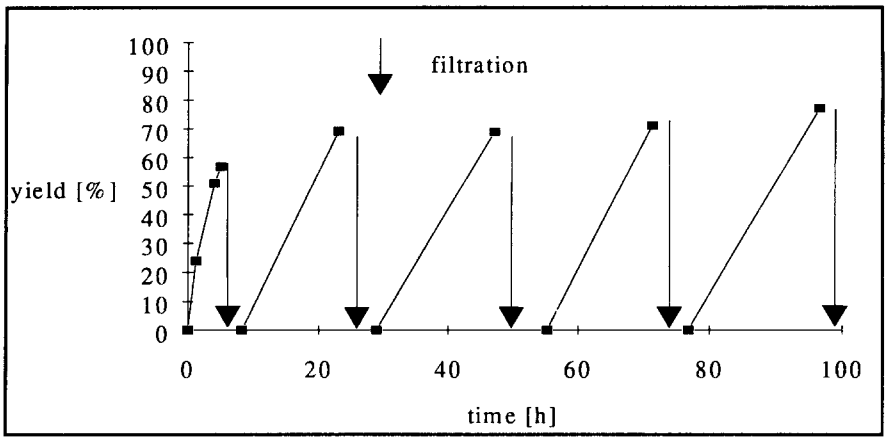


Fig. 1: Repetitive batch synthesis of dUDP-6-deoxy-α-D-*xylo*-4-hexulose **5**. Arrows indicate filtration steps.

Table 2 summarizes the results of the synthesis and the isolation of nucleotide sugars which were produced by this technique. The yields for product isolation are different reflecting the different sensitivity of the nucleotide(deoxy) sugars for decomposition. However, compared to our previous method[11] decomposition of nucleotides by alkaline phosphatase was very efficient with a 5 - 7% loss of activated sugars. In the course of our work we found that pyruvate could be more efficiently separated by ion exchange chromatography on Dowex 1x2, 100 -200 mesh, Cl^--form, rather than by Sepharose Q FF. A small amount of **3a** caused by the formation of **2a** in the kinase reaction (**A**) and appearing during the synthesis of **3b-d** can be separated by gel filtration. However, in the case of uridine-5′-diphosphate activated (deoxy) sugars adenosine-5′-triphosphate can be replaced by uridine-5′-triphosphate (UTP) in the kinase reaction (**A**) whereas cytidine-5′-triphosphate (CTP) and 2′-deoxyuridine-5′-triphosphate (dUTP) are not substrates of NMPK. The efficiency of the isolation protocol is especially demonstrated by the high yields of **3d** and **5**.

DISCUSSION

Sucrose synthase (**B**) catalyses the cleavage of sucrose **4** with UDP **2c**, dUDP **2d**, 2′-deoxythymidine-5′-diphosphate (dTDP), ADP **2a**, CDP **2b** and guanosine-5′-diphosphate (GDP)[10]. The continuous synthesis of 2′-deoxythymidine-5′-(α-D-glucopyranosyl)diphosphate (dTDP-Glc) in an enzyme membrane reactor demonstrated the suitability of sucrose synthase for the synthesis of nucleotide sugars on a preparative scale[11]. Although nucleoside diphosphates are commercially available they are still expensive substrates compared to nucleoside monophosphates. The utilization of nucleoside monophosphate kinase or myokinase (**A**) enabled us to produce **3a-c** in moderate and **3d** in good yields starting from their corresponding nucleoside monophosphates **1a-d** and **4**.

GDP-Glc could not be synthesized since GDP is a poor substrate for sucrose synthase[10]. However, nucleoside monophosphate kinase is able to catalyse the phosphorylation of guanosine-5′-monophosphate (GMP).[5]

2′-Deoxythymidine-5′-monophosphate (dTMP) was not a substrate of the commercial available kinases. In this case the phosphorylation of **1d** by NMPK[18] is a key step in the synthesis of nucleotide deoxysugars starting from **4** and NMPs in combination with a kinase (**A**), sucrose synthase (**B**) and dTDP-glucose 4,6-dehydratase (**D**).

Table 2: Nucleotide Sugar Syntheses using Repetitive Batch Technique.

NDP-sugar	**3a**	**3b**	**3c**	**3d**	**5**
			Enzymatic Synthesis		
synthezised (mg)	421	39	255	225	149
yield (%)	39	7	38	68	61
			Product Isolation		
starting material (mg)	233	39	251	225	149
isolated (mg)	63	7.5	141	162	120
yield (%)	27	19	57	72	80
Overall yield (%)	**10**	**1**	**21**	**49**	**49**

In addition, the repetitive batch technique is an efficient method for the synthesis of nucleotide sugars on a preparative scale using native and expensive enzymes. Table 3 shows that the costs (chemicals and enzymes without SuSy) for the synthesis of 1 g nucleotide sugar can be efficiently reduced.

Table 3: Estimated Costs (Chemicals and Enzymes) for Nucleotide Sugars Production without (Costs A) and with Repetitive Batch Technique (Costs B, 10 Batches)

NDP-Glc	costs A [$*g^{-1}]	costs B [$*g^{-1}]
3a	327	85
3b	4861	931
3c	2067	275
3d	8467	1046

EXPERIMENTAL

Materials

Sucrose synthase (**B**, SuSy) from rice grains and recombinant dTDP-Glc 4,6-dehydratase (**D**) (from *Salmonella enterica* group B) were purified as described elsewhere[17,19]. Nucleoside monophosphate kinase (NMPK, EC 2.7.4.4) from bovine liver, calf intestinal alkaline phosphatase (EC 3.1.3.1), pyruvate kinase (PK, EC 2.7.1.40) and lactate dehydrogenase (LDH, EC 1.1.1.28) from rabbit muscle were from Boehringer (Mannheim,

Germany). The nucleoside mono- and diphosphates, myokinase (MK, EC 2.7.4.3) from rabbit, chicken, porcine muscle and phospho(enol)pyruvate (PEP, Na salt) were supplied by Sigma (Deisenhofen, Germany). PEP (monocyclohexylammonium, CHA-salt) and NADH were from Biomol (Hamburg, Germany). All the other chemicals were purchased from Merck (Darmstadt, Germany).

Analytical methods

The nucleotide sugars and the nucleotides were analyzed by ion pair HPLC[20]. The activity of SuSy (**B**) was determined for the cleavage reaction with UDP[20]. The dTDP-glucose 4,6-dehydratase (**D**) activity was assayed with 2´-deoxythymidine-5´-(α-D-glucopyranosyl)diphosphate (dTDP-Glc) as substrate[13].

Photometric assay of kinases

The activity of different kinases with different nucleoside monophosphates (NMP) were determined with a photometric assay.

The assay mixture consisted of 5 mM NMP, 0.6 mM ATP, 0.8 mM PEP (Na salt), 7 U PK, 19.6 U LDH, 0.2 mM NADH, kinase (1 U MK bzw. 0.1 U NMPK) in buffer (0.1 M Tris(hydroxymethyl)aminomethane (Tris)-HCl pH 7.6, 1.2 mM $MgSO_4$, 0.14 M KCl, 1 mg*ml^{-1} bovine serum albumine (BSA)). The reaction was started by the addition of appropriate diluted enzyme solution. The final volume was 1 ml. The decrease of absorption was measured at 340 nm at 37° C. One unit enzyme is the amount of enzyme which produces 1 µmol product per minute.

pH-optima for the synthesis of nucleotide sugars

4 mM NMP, 6 mM PEP (Na salt), 6 mM $MgCl_2$, 0.2 U*ml^{-1} SuSy, 10 U*ml^{-1} PK and 0.05 U*ml^{-1} MK from rabbit muscle (substrate: **1a**) or 0.2 U ml^{-1} NMPK (substrate: **1d**) were mixed in 1 ml buffer (0.1 M Tris-HCl, 500 mM sucrose, 3 mM dithiothreitol (DTT), 1 mg*ml^{-1} BSA, pH: 7.0 - 8.0) and incubated for 17 h at 30° C. The reaction was stopped by separating the proteins from the substrate solution by an ultrafiltration with a centricon™10 (Amicon, Beverly U.S.A) and analysed by HPLC.

Preparative Synthesis of nucleotide sugars

For all syntheses we used the repetitive batch technique[16].

*1. 2´-Deoxyuridine-5´-(α-D-glucopyranosyl)diphosphate (**3d**)*

20 ml of 0.1 M Tris-HCl pH 7.2 with 4 mM **1d** (Na salt) (**I**), 6 mM PEP (Na salt) (**II**), 0.8 mM $MgCl_2$ (**III**), 0.12 mM ATP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 20 mg BSA was incubated with 40 U SuSy, 400 U PK and 20 U NMPK at 37 °C in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 30, cut-off 30.000 g*mol-1). After 4 hours the solution containing the product and non-reacted substrate was separated from the enzymes by ultrafiltration. The solution was concentrated to 2 ml. 18 ml fresh substrate solution containing compounds **I-VI** in the same concentration was added and treated like the first batch. Eight batches were performed and 0.409 mmol (225 mg, 68%) **3d** was prepared from 0.6 mmol **1d**.

The solution with 0.409 mmol dUDP-Glc was incubated with alkaline phosphatase (1 U/ml product solution) for 17 hours at 30° C. The enzyme was removed by ultrafiltration. The nucleotide sugar was purified by ion-exchange using Sepharose Q FF, Cl^--form (column: 2.6 x 35.4 cm, flow rate: 4 ml*min^{-1}). After sample loading

the column was rinsed with dest. water before the nucleotide sugar was eluted with a LiCl-gradient (0 - 0.3 M LiCl, V = 1 l). The solution was concentrated at 25 - 30° C and 20 - 25 mbar and desalted on Sephadex G 10 (column: 2.6 x 93 cm, flow rate: 1 ml*min^{-1}). 251 mg product (containing 162 mg **3d** and LiCl; HPLC: 98.3% **3d**, 0.9% **1d**, 0.8% 2´-deoxyuridine) were isolated after lyophilization. Yield: 0.294 mmol (72% based on 0.409 mmol **3d**).

^{1}H-NMR (360 MHz, D_2O): δ = 8.02 (d, 1H, H-6", $^3J_{H-5",H-6"}$ = 8 Hz); 6.41 (dd, 1H, H-1', $^3J_{H-1',H-2a,b'}$ = 7 Hz); 6.04 (d, 1H, H-5", $^3J_{H-5",H-6"}$ = 8 Hz); 5.68 (dd, 1H, H-1, $^3J_{H-1,H-2}$ = 3.4 Hz, $^3J_{H-1,\ P}$ = 6.8 Hz); 4.79 (HDO); 4.69 (m, 1H, H-3'); 4.31-4.22 (m, 3H, H-4', H-5'a,b); 4.01-3.94 (m, 1H, H-5); 3.93 (m, 1H, H-6a, $^2J_{H-6a,H-6b}$ = 12 Hz, $^3J_{H-5,H-6a}$ = 2.2 Hz); 3.88-3.81 (m, 2H, H-6b, H-4, $^2J_{H-6a,H-6b}$ = 12 Hz, $^3J_{H-5,\ H-6b}$ = 5 Hz, $^3J_{H-3,\ H-4}$ = 9 Hz, $^3J_{H-4,\ H-5}$ = 9Hz), 3.61 (ddd, 1H, H-2, $^3J_{H-2,H-3}$ = 10 Hz, $^3J_{H-1,H-2}$ = 3.2 Hz, $^4J_{H-2,P}$ = 3.2 Hz); 3.53 (dd, 1H, H-3, $^3J_{H-3,H-4}$ = 10 Hz, $^3J_{H-2,H-3}$ = 10 Hz), 2.48 (m, 2H, H-2a,2b)

^{13}C-NMR (75 MHz, D_2O): δ = 168.7 (s, C-4"); 154.1 (s, C-2"); 144.3 (s, C-6"), 104.9 (s, C-5"); 98.0 (d, C-1, $^2J_{C-1,P}$ = 6.8 Hz), 87.9 (d, C-4', $^3J_{C-4',P}$ = 9.0 Hz); 87.8 (s, C-1'); 75.3 (s, C-4); 75.2 (s, C-5); 74.1 (d, C-2, $^3J_{C-2,P}$ = 8.3 Hz); 73.3 (s, C-3'); 71.7 (s, C-3); 67.9 (d, C-5', $^2J_{C-5',P}$ = 6.0 Hz); 62.8 (s, C-6); 41.3 (s, C-2')

ESI-MS (negative mode) m/z = 549 $[M-H]^-$; m/z = 555 $[M(Li)-H]^-$

2. Uridine-5´-(α-D-glucopyranosyl)diphosphate (3c)

50 ml of 0.1 M Tris-HCl pH 7.2 with 4 mM **1c** (Na salt) (**VII**), 6 mM PEP (CHA-salt) (**II**), 3.2 mM $MgCl_2$ (**III**), 0.12 mM UTP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 50 mg BSA was incubated with 6.3 U SuSy, 1000 U PK and 5 U NMPK at 30 °C in a stirred ultrafiltration cell as described above. After 21 hours the solution was concentrated to 5 ml. 45 ml fresh substrate solution containing compounds **II, III, V, VI** and **VII** in the same concentration was added and treated like the first batch. Six batches were performed with incubation periods between 19.5 h and 27 h. 0.451 mmol (255 mg, 38%) **3c** were synthesized from 1.2 mmol **1c**.

0.444 mmol **3c** was taken for product isolation as described above. 402 mg product (containing 141 mg **3c**, pyruvate and LiCl, HPLC: 92.6% **3c**, 5.4% **1c**, 2.0% Uridine) were isolated. Yield: 0.253 mmol, 57% based on 0.444 mmol **3c**. For NMR analysis a part of isolated **3c** was purified again by Sepharose Q FF, concentration and gelfiltration. 22.8 mg **3c** (61% w/w) were isolated.

^{1}H-NMR (300 MHz, D_2O): δ = 7.96 (d, 1H, H-6", $^3J_{H-5",H-6"}$ = 8 Hz); 5.99 (d, 1H, H-1', $^3J_{H-1',H-2'}$ = 5 Hz); 5.99 (d, 1H, H-5", $^3J_{H-5",H-6"}$ = 8 Hz); 5.61 (dd, 1H, H-1, $^3J_{H-1,H-2}$ = 3.6 Hz, $^3J_{H-1,\ P}$ = 7.6 Hz); 4.79 (HDO); 4.39 (m, 2H, H-2', H-3'); 4.3 (m, 1H, H-4'); 4.24 (m, 2H, H-5'a,b); 3.94-3.87 (m, 1H, H-5); 3.87 (m, 1H, H-6a, $^2J_{H-6a,H-6b}$ = 12.4 Hz, $^3J_{H-5,H-6a}$ = 2.4 Hz); 3.83-3.73 (m, 2H, H-6b, H-4, $^2J_{H-6a,H-6b}$ = 12.4 Hz, $^3J_{H-5,\ H-6b}$ = 5 Hz, $^3J_{H-3,\ H-4}$ = 9 Hz, $^3J_{H-4,\ H-5}$ = 9 Hz), 3.55 (ddd, 1H, H-2, $^3J_{H-2,H-3}$ = 9.6 Hz, $^3J_{H-1,H-2}$ = 3.6 Hz, $^4J_{H-2,P}$ = 3.2 Hz); 3.47 (dd, 1H, H-3, $^3J_{H-3,H-4}$ = 9 Hz, $^3J_{H-2,H-3}$ = 10 Hz)

^{13}C-NMR (75 MHz, D_2O): 169.1 (s, C-4"); 154.7 (s, C-2"); 144.5 (s, C-6"), 105.5 (s, C-5"); 98.4 (d, C-1, $^2J_{C-1,P}$ = 6.7 Hz), 91.3 (s, C-1'), 86.1 (d, C-4', $^3J_{C-4',P}$ = 9.3 Hz); 76.6 (s, C-2'); 75.7 (s, C-4); 75.7 (s, C-5); 74.4 (d, C-2, $^3J_{C-2,P}$ = 8.7 Hz); 72.5 (s, C-3'); 72.0 (s, C-3); 67.8 (d, C-5', $^2J_{C-5',P}$ = 5.6 Hz); 63.2 (s, C-6)

*3. Cytidine-5´-(α-D-glucopyranosyl)diphosphate (**3b**)*

50 ml of 0.1 M Tris-HCl pH 7.5 containing 4 mM **1b** (Na salt) (**VIII**), 6 mM PEP (CHA-salt) (**II**), 3.2 mM $MgCl_2$ (**III**), 0.12 mM ATP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 50 mg BSA was incubated with 50 U SuSy, 1000 U PK and 500 U MK at 30 °C in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 10, cut-off 10.000 g*mol^{-1}) . After incubation the solution was concentrated to 5 ml and the reaction was started with 45 ml of a solution containing compounds **II-VI** and **VIII.** Five batches were performed with incubation periods between 19 h and 24 h. 0.069 mmol (39 mg, 7%) **3b** were prepared from 1 mmol **1b**.

0.069 mmol **3b** were isolated as described above yielding 9.2 mg product (81% w/w **3b**, HPLC: 89% **3b**, 11% **1b**). Yield: 0.013 mmol, 19% based on 0.069 mmol **3b**.

^{1}H-NMR (360 MHz, D_2O): δ = 8.02 (d, 1H, H-6", $^3J_{H\text{-}5",H\text{-}6"}$ = 8 Hz); 6.2 (d, 1H, H-5", $^3J_{H\text{-}5",H\text{-}6"}$ = 8.0 Hz); 6.07 (d, 1H, H-1', $^3J_{H\text{-}1',H\text{-}2'}$ = 4.5 Hz); 5.66 (dd, 1H, H-1, $^3J_{H\text{-}1,H\text{-}2}$ = 3.6 Hz, $^3J_{H\text{-}1,\,P}$ = 7.4 Hz); 4.78 (HDO); 4..44-4.23 (m, 5H, H-2', H-3', H-4', H-5'a,b); 4.0-3.92 (m, 1H, H-5); 3.92 (m, 1H, H-6a, $^2J_{H\text{-}6a,H\text{-}6b}$ = 12.2 Hz, $^3J_{H\text{-}5,H\text{-}6a}$ = 2.4 Hz); 3.88-3.8 (m, 2H, H-6b, H-4, $^2J_{H\text{-}6a,H\text{-}6b}$ = 12.2 Hz, $^3J_{H\text{-}5,\,H\text{-}6b}$ = 4.2 Hz), 3.61 (ddd, 1H, H-2, $^3J_{H\text{-}2,H\text{-}3}$ = 10.6 Hz, $^3J_{H\text{-}1,H\text{-}2}$ = 3.5 Hz, $^4J_{H\text{-}2,P}$ = 3.3 Hz); 3.54 (dd, 1H, H-3, $^3J_{H\text{-}3,H\text{-}4}$ = 9.2 Hz, $^3J_{H\text{-}2,H\text{-}3}$ = 9.8 Hz)

^{13}C-NMR (90 MHz, D_2O): 168.7 (s, C-4"); 160.3 (s, C-2"); 143.9 (s, C-6"), 99.1 (s, C-5"); 98.0 (d, C-1, $^2J_{C\text{-}1,P}$ = 6.9 Hz), 91.7 (s, C-1'), 85.2 (d, C-4', $^3J_{C\text{-}4',P}$ = 8.4 Hz); 76.7 (s, C-2'); 75.4 (s, C-4); 75.2 (s, C-5); 74.1 (d, C-2, $^3J_{C\text{-}2,P}$ = 8.4 Hz); 71.8 (s, C-3'); 71.7 (s, C-3); 67.2 (d, C-5', $^2J_{C\text{-}5',P}$ = 5.3 Hz); 62.9 (s, C-6)

*4. Adenosine-5´-(α-D-glucopyranosyl)diphosphate (**3a**)*

100 ml of 0.1 M Tris-HCl pH 7.5 containing 4 mM **1a** (Na salt) (**IX**), 6 mM PEP (CHA-salt) (**II**), 8 mM $MgCl_2$ (**III**), 0.12 mM ATP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 100 mg BSA was incubated with 100 U SuSy, 1000 U PK and 50 U MK at 37 °C. After the incubation time of 4 hours the solution was concentrated to 10 ml in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 10, cut-off 10.000 g*mol^{-1}) and the reaction was started with 190 ml of a solution containing compounds **II-VI** and **IX**. Three batches were performed with incubation periods between 4 h and 17 h resulting in the synthesis of 0.717 mmol (421 mg, 39%) **3a** from 1.84 mmol **1a**.

0.397 mmol **3a** was treated with alkaline phosphatase, ultrafiltrated and isolated by Sepharose Q FF as described above. Residual pyruvate in the preparation of **3a** was removed by ion-exchange (Dowex 1x2, Cl^--form, 100 - 200 mesh, 2.6 x 35.4 cm; flow rate: 4 ml*min^{-1}; gradient: 0 - 0.5 M LiCl, V = 0.5 l; **3a** was eluted with 1 M LiCl). After concentration, desalting and lyophilisation 76.7 mg product (82% **3a** w/w, HPLC: 99% **3a**) were isolated. Yield: 0.107 mmol, 27% based on 0.397 mmol **3a**.

^{1}H-NMR (300 MHz, D_2O): δ = 8.5 (s, H-8"); 8.23 (s, H-2"); 6.1 (d, 1H, H-1', $^3J_{H\text{-}1',H\text{-}2'}$ = 6 Hz); 5.58 (dd, 1H, H-1, $^3J_{H\text{-}1,H\text{-}2}$ = 3.6 Hz, $^3J_{H\text{-}1,\,P}$ = 7.6 Hz); 4.78 (m, 1H, H-2'); 4.74 (HDO); 4.55 (m, 1H, H-3'); 4.41 (m, 1H, H-4'); 4.25 (m, 2H, H-5'a,b); 3.93-3.86 (m, 1H, H-5); 3.85 (m, 1H, H-6a, $^2J_{H\text{-}6a,H\text{-}6b}$ = 12.8 Hz); 3.80-3.74 (m, 2H, H-6b, H-4, $^2J_{H\text{-}6a,H\text{-}6b}$ = 12.8 Hz, $^3J_{H\text{-}5,\,H\text{-}6b}$ = 4.8 Hz), 3.5 (ddd, 1H, H-2, $^3J_{H\text{-}2,H\text{-}3}$ = 9.5 Hz, $^3J_{H\text{-}1,H\text{-}2}$ = 3.3 Hz, $^4J_{H\text{-}2,P}$ = 3.0 Hz); 3.43 (dd, 1H, H-3, $^3J_{H\text{-}3,H\text{-}4}$ = 9.0 Hz, $^3J_{H\text{-}2,H\text{-}3}$ = 9.5 Hz)

^{13}C-NMR (75 MHz, D_2O): 158.5 (s, C-6"); 155.8 (s, C-2"); 152.0 (s, C-4"), 142.7 (s, C-8"); 121.5 (s, C-5"); 98.4 (d, C-1, $^2J_{C\text{-}1,P}$ = 6.6 Hz), 89.7 (s, C-1'), 86.7 (d, C-4', $^3J_{C\text{-}4',P}$ = 9.3 Hz); 77.1 (s, C-2') ; 75.7 (s, C-4);

75.66 (s, C-5); 74.5 (d, C-2, $^3J_{C-2,P}$ = 8.5 Hz); 73.2 (s, C-3'); 72.1 (s, C-3); 68.1 (d, C-5', $^2J_{C-5',P}$ = 5.6 Hz); 63.2 (s, C-6)

5. dUDP-6-deoxy-α-D-xylo-4-hexulose (5)

25 ml of 0.1 M Tris-HCl pH 7.2 with 4 mM **1d** (Na salt) (**I**), 6 mM PEP (Na salt) (**II**), 0.8 mM $MgCl_2$ (**III**), 0.12 mM ATP (Na salt) (**IV**), 500 mM **4** (**V**), 3 mM DTT (**VI**) and 25 mg BSA was incubated with 50 U SuSy, 500 U PK, 25 U NMPK and 125 U dTDP-Glc-4,6-dehydratase at 25 °C in a stirred ultrafiltration cell (Amicon, Model 8050, equipped with a membrane YM 10, cut-off 10.000 g*mol^{-1}). After 5 h the solution was concentrated to 2.5 ml and 22.5 ml fresh substrate solution containing compounds **I-VI** in the same concentration was added and treated like the first batch. Five batches were performed with incubation periods between 5 h and 20 h. 0.279 mmol (149 mg, 61%) **5** were prepared from 0.456 mmol **1d**.

0.279 mmol was purified by ion-exchange (Dowex 1x2, Cl^--form, as described ADP-Glc), concentrated, desalted and lyophilized. 241 mg product (containing 120 mg **5** and LiCl, HPLC: 93%) were isolated. Yield: 0.224 mmol, 80% based on 0.279 mmol **5** (hydrate : ketoform: 4.5 : 1)

Hydrate:

^{1}H-NMR (300 MHz, D_2O) δ = 7.84 (d, 1 H, H-6", $^3J_{H-5",\ H-6"}$ = 8 Hz), 6.22 (dd, 1 H, H-1', $^3J_{H-1',\ H-2'a} \approx {}^3J_{H-1',\ H-2'b}$ = 7 Hz), 5.86 (d, 1 H, H-5", $^3J_{H-5",\ H-6"}$ = 8 Hz), 5.45 (dd, 1 H, H-1, $^3J_{H-1,\ P}$ = 6.8 Hz, $^3J_{H-1,\ H-2}$ = 3.4 Hz), 4.64 (HDO), 4.5 (m, 1 H, H-3'), 4.14 - 4.06 (m, 3H, H-5'a,b, H-4'), 4.0 (q, 1 H, H-5, $^3J_{H-5,\ H-6}$ = 6.2 Hz), 3.69 (d, 1 H, H-3, $^3J_{H-2,\ H-3}$ = 10 Hz), 3.55 (ddd, 1 H, H-2, $^3J_{H-2,\ H-3}$ = 10.2 Hz, $^3J_{H-1,\ H-2}$ = 3.4 Hz, $^4J_{H-2,\ P}$ = 3.4 Hz), 2.36 - 2.22 (m, 2 H, H-2'a,b), 1.13 (d, 3 H, H-6, $^3J_{H-5,\ H-6}$ = 6.2 Hz)

^{13}C-NMR (75 MHz, D_2O) δ = 166.9 (s, C-4"), 152.2 (s, C-2"), 142.4 (s, C-6"), 103.1 (s, C-5"), 95.9 (d, C-1, $^2J_{C-1,\ P}$ = 6.6 Hz), 94.3 (s, C-4), 86.005 (d, C-4', $^3J_{C-4',\ P}$ = 8.7 Hz), 85.996 (s, C-1'), 73.7 (s, C-3), 71.4 (s, C-3'), 71.1 (d, C-2, $^3J_{C-2,\ P}$ = 8.7 Hz), 70.2 (s, C-5), 66.0 (d, C-5', $^2J_{C-5',\ P}$ = 5.6 Hz), 39.4 (s, C-2'), 11.9 (s, C-6)

Keto form:

^{1}H-NMR (300 MHz, D_2O) δ = 5.64 (dd, 1 H, H-1, $^3J_{H-1,\ P}$ = 7.2 Hz, $^3J_{H-1,\ H-2}$ = 3.4 Hz), 3.75 (ddd, 1 H, H-2, $^3J_{H-2,\ H-3}$ = 10.2 Hz, $^3J_{H-1,\ H-2}$ = 3.4 Hz, $^4J_{H-2,\ P}$ = 3.4 Hz), 1.17 (d, 3 H, H-6, $^3J_{H-5,\ H-6}$ = 6.4 Hz)

^{13}C-NMR (75 MHz, D_2O) δ = 206.5 (s, C-4), 95.6 (d, C-1, $^2J_{C-1,\ P}$ = 6.6 Hz), 76.0 (s, C-3), 75.3 (d, C-2, $^3J_{C-2,\ P}$ = 8.8 Hz), 70.7 (s, C-5), 13.4 (s, C-6)

^{31}P-NMR (121 MHz, D_2O) δ = -11.85 (d, 1 P, Pα), -13.65 (d, 1 P, Pß), $^2J_{P\alpha,\ P\beta}$ = 19 Hz

ESI-MS (negative mode): m/z = 532 $[M(ketoform)-H]^-$; m/z = 550 $[M(hydrate)-H]^-$

ACKNOWLEDGEMENTS

This work is a part of the PhD thesis of Dipl.-Ing. (FH) Dipl. Chem. Astrid. Zervosen. The financial support by the Hoechst AG, Frankfurt am Main, and the excellent technical assistance of Mrs. Birgit Güldenberg is gratefully acknowledged. We thank Prof. Dr. M.-R. Kula for helpful discussions. The authors also thank Prof.

W. Piepersberg, Dr. J. Distler and Dipl.-Biol. Stefan Verseck, Institute of Chemical Microbiology, University Wuppertal, for providing us with an *Escherichia coli* strain containing recombinant dTDP-Glc 4,6-dehydratase.

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(*Received in Germany* 25 *October* 1995; *revised* 10 *December* 1995; *accepted* 12 *December* 1995)