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Combined preparative enzymatic synthesis of dTDP-6-deoxy-4-keto-D-glucose from dTDP and sucrose

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dTDP-6-deoxy-4-keto-D-glucose (1), the common intermediate in the biosyntheses of the manifold deoxysugars, was synthesized on a gram-scale by the combination of sucrose synthase and dTDP-D-glucose 4,6-dehydratase in a fed batch, starting the reaction with dTDP. This process allowed a dTDP conversion with a 100% rate. An easy and efficient three-step purification with anion-exchange chromatography and gel filtration gave 1.1 g of 1 in an overall yield of 73%. This work realizes a first step for an economic access to activated deoxysugars.

***Keywords*: activated deoxysugars, preparative enzymatic synthesis, sucrose synthase, dehydratase**

Introduction

A large variety of deoxysugars is found as building blocks in the oligosaccharide moieties of glycoproteins, glycolipids, and different classes of secondary metabolites, essentially determining their roles in intra- and intercellular communication [1–5]. Investigation of structure-function relations or the alteration of glycosylation patterns are largely based on the availability of adequate carbohydrate structures. Glycosyltransferases of the Leloir pathway constitute a powerful tool to accomplish the synthesis of oligosaccharides [6, 7]. Aiming at deoxysugars in particular, nucleotide deoxysugars must be supplied as substrates for the corresponding glycosyltransferases. dTDP-6-deoxy-4-keto-D-glycose (**1**) is the common intermediate in the biosynthesis of all dTDP-deoxysugars, forming the starting-point for a broad-fanned branching (Figure 1). From here on, the transition to the L-sugars takes place and in a series of succeeding reactions, all utilizing activated intermediates, the various deoxygenated, aminated, and branched sugars that are found in antibiotics or bacterial cell walls arise. The synthesis and NMR-spectroscopic characterization of **1** was recently reported enzymatically converting dTDP-D-glucose with dTDP-D-glucose 4,6-dehydratase [8–10]. Nevertheless, starting the synthesis at this point is not economic because the nucleotide sugar is very expensive and furthermore requires a highly selective purification step to remove impurities from the commercial preparation. Sucrose synthase was previously applied using an enzyme-membrane reactor to yield dTDP-D-glucose on a preparative scale starting from dTDP and sucrose [11]. In the present paper we extend this approach for the synthesis of **1** on a gram-scale by the combination of partially purified sucrose synthase and dTDP-D-glucose 4,6-dehydratase in a fed batch process (Figure 2).

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Materials and methods

Materials

The clone of dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46) from *Salmonella typhimurium* LT2 expressed in *E. coli* BL21 was a kind gift from Professor Piepersberg (University of Wuppertal, Germany). The dehydratase gene was under the control of the bacteriophage T7 promotor in a pT7-6 expression vector. Enzyme production has been carried out as described for the overexpression of other enzymes using the same vector system [12].

Sucrose synthase (UDPG:D-fructose-2-glucosyltransferase EC 2.4.1.13) from rice grains was purified on pilot scale according to [17]. The enzyme assay and most of the enzyme's properties have been reported previously [13, 14]. Maxatase was a product from Gist Brocades (Delft, The Netherlands), dTDP and dTDP-D-glucose were purchased from Sigma (Deisenhofen, Germany).

Enzyme isolation and characterization

The activity of dTDP-D-glucose 4,6-dehydratase was assayed spectrophotometrically via product formation [15] or

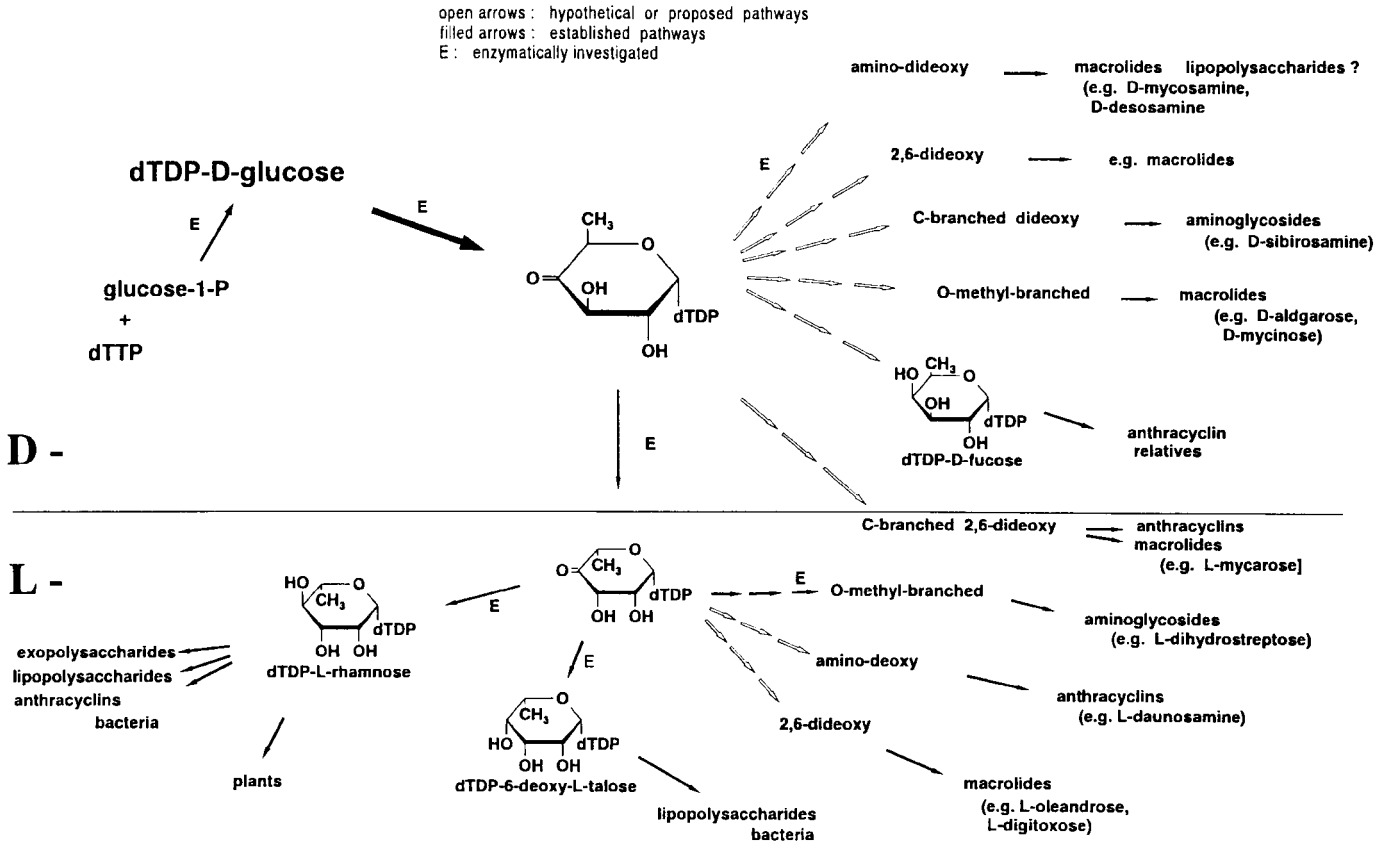

Figure 1. Biosynthesis of dTDP-activated deoxysugars.

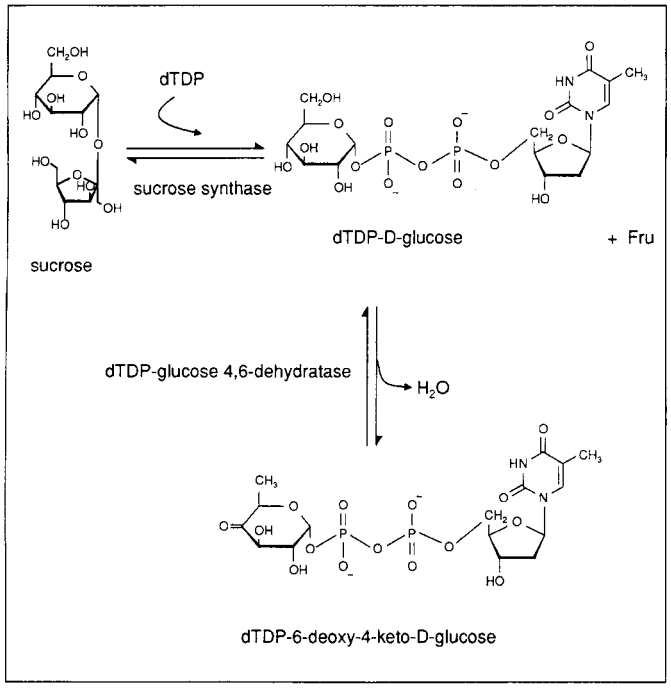

Figure 2. Reaction scheme for the combined synthesis.

by ion pair HPLC [16, 9] detecting the decrease of dTDP-D-glucose. To detect proteases 350 µl azo caseine solution (prepared from 0.5 g azo caseine dissolved in 25 ml 0.1 M phosphate buffer, pH 6.8), 50 µl crude extract and 370 µl 0.1 M phosphate buffer, pH 6.8 were incubated for 15 min at 37 °C. After the addition of 350 µl trichloroacetic acid the precipitate was centrifuged off, 770 µl of the supernatant were mixed with 230 µl 5 M sodium hydroxide solution, and the absorption measured at 440 nm. Blanks were performed replacing the crude extract by 50 mM Tris/HCl buffer, pH 7.5. A calibration curve was prepared conducting the test with 0–2 U ml^{-1} of maxatase (commercial protease preparation). Invertase was assayed as described earlier [17]. Phosphatases were measured according to [14] and expressed as volume activities. Nucleotide sugar cleavage was analysed by incubating dTDP-6-deoxy-4-keto-D-glucose (1.18 mg, 2 mM) in 50 mM Tris/HCl, pH 7.5 with 20 µl crude extract at 30 °C. The final volume was 500 µl. Blanks were carried out with denatured enzyme solution. After zero and 25 h the solutions were analysed by HPLC.

To separate the dehydratase from contaminating protease activities, the crude extract was subjected to an anion-exchange chromatography on Q-Sepharose FF (Cl^--form).

At room temperature the column (2.6 cm × 8 cm, 43 ml wet gel, 5 ml min^{-1} flow rate) was equilibrated with 50 mM Tris/HCl, pH 7.5, containing 0.2 M KCl. The gel was loaded with up to 25 ml of crude extract (maximum amount of protein 200 mg, 2.5 ml min^{-1} flow rate) and washed with the Tris/HCl buffer. When the UV absorbance at 280 nm was about zero the enzyme was eluted with a linear KCl gradient (0.2–0.35 M) in Tris/HCl (575 ml, 10 ml per fraction). Active fractions were detected by the spectrophotometric assay and combined. All characterization of the dehydratase was conducted after the purification step. The pH-optimum was investigated in 200 mM HEPES: 350 mM sucrose: 1 mg ml^{-1} BSA at 37 °C, varying the pH from 7.0 to 8.5.

The pH-stability was examined between pH 7.0 and pH 8.5, incubating the enzyme (112 U ml^{-1}) under sterile conditions in 200 mM HEPES: 350 mM sucrose: 1 mg ml^{-1} BSA at 25 °C. The temperature-stability was ascertained at 25 and 37 °C at pH 7.0 as described for pH-stability, adding various potential stabilizers. For that purpose an aliquot of the enzyme pool was diluted 1:5 into the stabilizer solutions and the remaining activity assayed after defined intervals. The deactivation constants k_{des} were deduced from the curves of temperature- and pH-stability, assuming a first order reaction.

The kinetics for both the dTDP-D-glucose 4,6-dehydratase and the sucrose synthase were measured by the initial reaction rates, allowing conversions between 3 and 6%. Describing the dehydratase, dTDP-D-glucose was varied between 5 and 100 µM. Further, two higher concentrations, 1 and 3 mM, were included to uncover possible substrate inhibition. The assay mixtures were incubated for 15 min at 25 °C (20 mM HEPES: 1 mg ml^{-1} BSA, pH 8.0) and the reactions stopped by heating for 5 min at 95 °C. Studying the cross inhibition by nucleoside diphosphate, a concentration of between 0.03 and 3 mM of dTDP-D-glucose was chosen in the presence of 0.02 mM dTDP. The mixtures were analysed either photometrically or by HPLC, each assay being sensitive down to concentration changes of 6 and 1 µM respectively.

The kinetic behaviour of sucrose synthase was thoroughly investigated with dTDP as substrate. Altering the nucleoside diphosphate concentration between 0.1 and 10 mM, the assay mixtures were incubated for 40 min at 30 °C (200 mM HEPES: 350 mM sucrose, pH 7.2). The reactions were stopped by heating for 5 min at 95 °C and the enzyme activity subsequently determined by HPLC. A potential inhibition of sucrose synthase by dTDP-6-deoxy-4-keto-D-glucose was examined with two tests. Both of them contained 0.025 mM of UDP as substrate, one additionally including 2 mM of the deoxysugar.

For both enzymes the kinetic constants were calculated from direct Michaelis-Menten plots by the Marquardt non-linear regression method using the graphic and statistic computer program PloIt® (Scientific Programming Enterprises, Haslett, USA).

Table 1. Tests to examine the influence of enzyme ratio and concentration. The activities of the applied enzyme solutions were measured in 200 mM HEPES buffer: 1 mg ml^{-1} BSA, pH 7.0 at 25 °C.

Test	*Enzyme amount per ml test [U]*	
	Dehydratase	*Sucrose synthase*
1	0.11	0.11
2	0.55	0.11
3	0.55	0.33

Influence of enzyme amounts on the conversion rate

In order to obtain an optimum conversion throughout the combined synthesis different ratios of sucrose synthase and dTDP-D-glucose 4,6-dehydratase were reacted with the nucleoside diphosphate. As illustrated in Table 1 equal portions of both enzymes were applied in the first test. Secondly, the effect of a five-fold quantity of dehydratase was studied. The third experiment coupled this high enzyme level with the three-fold raised original amount of sucrose synthase. All the tests were performed in 200 mM HEPES buffer: 350 mM sucrose: 1 mg ml^{-1} BSA, pH 7.0 at 25 °C, containing 10 mM dTDP. The concentrations of dTDP, dTDP-D-glucose, and dTDP-6-deoxy-4-keto-D-glucose were monitored by HPLC. The corresponding enzyme activities were determined from the first derivatives of the concentration-time curves.

Preparative combined synthesis as fed batch

The synthesis was carried out in a stirred 50 ml ultrafiltration cell equipped with an Amicon YM10 membrane (cut-off 10 kDa) at 25 °C. All solutions had been sterilized by filtration. Eighty-one U of dTDP-D-glucose 4,6-dehydratase (after anion-exchange chromatography) and 53 U of partially purified sucrose synthase in 45 ml 200 mM HEPES buffer: 350 mM sucrose: 1 mg ml^{-1} BSA, pH 7.0 were filled in first. The reaction was started by adding dTDP (222.6 mg, 0.5 mmol) in 1 ml buffer (without BSA). Another 1.85 mmol of the nucleoside diphosphate was added in four portions, each after 1 h.

Isolation of dTDP-6-deoxy-4-keto-D-glucose

After the complete conversion of dTDP to dTDP-6-deoxy-4-keto-D-glucose the product solution was separated from protein by ultrafiltration. The membrane was rinsed with aq. bidest, and the combined filtrates were diluted in the ratio 1:10 to a salt concentration of about 10 mM. dTMP, a contaminant of commercial dTDP, was removed from the nucleotide deoxysugar by anion-exchange, using Dowex 1 × 2, Cl^- -form (2.6 cm × 22 cm column, 117 ml wet gel, 5 ml min^{-1} flow rate). After sample loading the column was

rinsed with aq. bidest, before the product was eluted with a linear NaCl gradient (0–0.5 M, 500 ml, 10 ml per fraction). The pooled fractions were concentrated by evaporation ($\leqslant$ 30 °C, oil pump vacuum) and the resulting small precipitate even dissolved by adding aq. bidest.

Desalting was carried out at 4 °C on Sephadex G-10 (2.6 cm × 93 cm column, 494 ml wet gel, 1 ml min^{-1} flow rate). Up to 1 g (1.7 mmol) dTDP-6-deoxy-4-keto-D-glucose and about 2.5 g NaCl in a maximum volume of 20 ml were loaded onto the column. Employing samples with such a high salt content required a low flow rate of 0.5 ml min^{-1} to prevent an irreversible gel compression. The product solution was evaporated down to 10 ml as described, frozen at –80 °C, and lyophilized at 15 °C. The isolated deoxysugar was characterized by NMR-spectroscopy and ESI-MS [9].

Results and discussion

Enzyme isolation and characterization

The production of dTDP-D-glucose 4,6-dehydratase from *Salmonella typhimurium* LT2 was enhanced 300-fold by overexpression in *E. coli* BL21, yielding crude extracts with a specific activity of 1.58 U mg^{-1}. Marumo *et al.* achieved a comparable result with a similar system that was induced by temperature [8]. The crude extract of our strain contained very little contaminating protease and phosphatase activity not being relevant to the stability of the educt or product during the combined synthesis (Table 2). The crude extract could be used successfully for enzymatic synthesis due to the overexpression of dTDP-D-glucose 4,6-dehydratase [9]. The contaminating phosphatases could then be neglected, degrading only 0.67% of the dTDP during the preparative reaction. A calculation for the wild type cells revealed that the activity of phosphatases would make up to 25% of sucrose synthase activity if the respectively larger amount of crude extract was applied. In this case degradation of dTDP would severely compete with conversion. However, for characterization studies recombinant dTDP-glucose 4,6-dehydratase was purified in an anion-exchange chromatography step by a factor of 4.4 and a yield of 81% removing all contaminating activities (Table 2).

All further characterization was carried out in 200 mM HEPES which conferred maximum activity and stability to

Table 2. Contaminating enzymes in dehydratase preparations.

Contaminating activity	*Crude extract (U ml^{-1})*	*Q Seph FF pool (U ml^{-1})*
Protease	0.013	0.0031
Invertase	0	0
Phosphatase	0.004	0
Nucleotide sugar cleavage	0	0

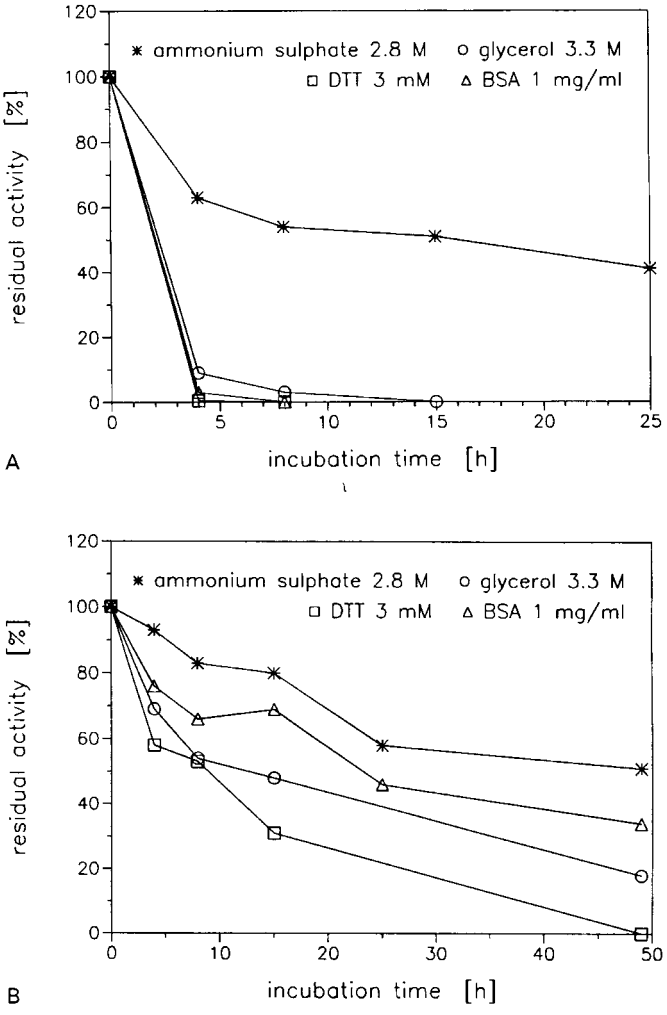

Figure 3. Temperature-stability of dTDP-D-glucose 4,6-dehydratase with different additives. (A) incubation at 37 °C, (B) incubation at 25 °C.

sucrose synthase [14], being also compatible with dTDP-D-glucose 4,6-dehydratase. After 48 h storage of purified dehydratase at 0 °C no loss of activity could be detected while in the crude extract activity decayed (1% per hour). This fact possibly corresponded to the reduction in protease activity.

dTDP-D-glucose 4,6-dehydratase had a broad pH-optimum of between pH 7.7 and pH 8.3. The enzyme's long term stability was investigated to guarantee that the catalyst was exploited efficiently. Ammonium sulphate as a stabilizer of dehydratase activity showed 60% residual activity after 4 h at 37 °C (Figure 3A) and 80% after 15 h at 25 °C (Figure 3B). It is an unpractical agent for product isolation however, due to the high salt concentration. Alternatively, a good operational stability was ensured, by employing 1 mg ml^{-1} BSA as a stabilizer along with the moderate temperature of 25 °C (Figure 3B). Table 3 summarizes the constants and half-life periods of enzyme deactivation for different temperatures and pH values.

Table 3. Constants and half-life periods of dehydratase deactivation

Conditions	k_{des} [%/d]	$t_{1/2}$ [h]
37 °C; pH 7.0	34.7	2
25 °C; pH 7.0	2.0	34
25 °C; pH 8.0	0.6	116

The dehydratase kinetics followed a simple Michaelis-Menten mechanism, having a V_{max} of 4.4 U mg^{-1} and a K_m for dTDP-D-glucose of 7.2 μM. dTDP was a strong inhibitor competitive to dTDP-D-glucose with a K_i of 2.5 μM. No evidence for substrate or product inhibition was found.

The calculated K_m for the enzyme from *Salmonella enterica* is distinctly smaller than all data so far reported. Those values ranged from 27 to 70 μM independent of the bacterial species and were all derived by using the spectrometric assay [18–22]. We obtained all kinetic data using the HPLC method which proved to be more sensitive for the detection of educt conversion down to 1 μM. In comparison, the spectrometric assay gave only reliable results down to 6 μM educt conversion. The consequence is that the initial rates obtained by the spectrometric assay are all too low probably resulting in higher K_m values. From our experiment it may be inferred that the dehydratase from *Salmonella enterica* is inhibited more strongly by dTDP than the enzymes from *E. coli* (K_i = 200 μM, [23]) or *Streptomyces rimosus* (K_i = 65 μM, [20]). The lack of both substrate and product inhibition was also described for the dehydratase from *E. coli* [24].

Sucrose synthase showed a K_m for dTDP of 0.16 mM. The K_i for substrate inhibition by dTDP was determined as 15 mM. The V_{max} under standard conditions (200 mM HEPES, pH 7.2 at 30 °C) with dTDP as substrate was 5.7 U mg^{-1}. In the presence of 2 mM dTDP-6-deoxy-4-keto-D-glucose sucrose synthase exhibited a residual activity of 83 ± 13%.

The enzyme showed an optimum pH of 6.8 for formation of dTDP-glucose and a relatively high temperature stability [14]. The half change values for inactivation at pH 7.0 were determined as 94 h at 37 °C and 119 h at 30 °C [13].

Reaction conditions for the combined synthesis

200 mM HEPES: 350 mM sucrose: 1 mg ml^{-1} BSA was chosen as buffer. The pH was adjusted to 7.0 with hydrochloric acid and the reaction carried out at the moderate temperature of 25 °C. These data constituted an effective compromise and were derived from the optimum conditions of the two individual reactions. Crucial for selecting the conditions were the different pH optima of both enzymes as well as the strong pH and temperature dependence of the dehydratase stability. While a pH value of 7.0 corresponded to the optimum activity and stability of sucrose synthase it impaired the activity and stability of the dehydratase. At neutral point the latter enzyme displayed a residual activity of 70% and a 3.5-times lower stability compared to the optimum pH of around 8. However, by setting the reaction temperature down to 25 °C an acceptable half change value for inactivation of 34 h was attained, distinct from only 2 h at 37 °C. The remaining lower level of dehydratase activity throughout a synthesis at pH 7.0 could easily be compensated by fresh enzyme, due to its overexpression. The temperature influence on the activity and stability of the dehydratase exemplifies the fact that the activation energy of an enzymatic reaction is far below the activation energy for thermal denaturation. Hence, by lowering the temperature the inactivation rate decreases more rapidly than the enzyme activity and more product is formed during the mean lifetime of the enzyme [25]. The hitherto published syntheses of small amounts of dTDP-6-deoxy-4-keto-D-glucose starting with dTDP-D-glucose were with few exceptions [20, 26, 27] carried out at 37 °C [8, 15, 28–33].

Influence of enzyme amounts on the conversion rate

In preliminary experiments an optimum conversion for the combined synthesis should be established. Figure 4 shows that both sucrose synthase and dTDP-D-glucose 4,6-dehydratase displayed only a small part of their maximum activity under synthesis conditions. Sucrose synthase activity decreased steeply at the beginning (Figure 4A), because of the product inhibition of dTDP-D-glucose (K_{ip} = 0.73 mM [13]). The dehydratase activity remained low due to the strong inhibition by dTDP, limiting the synthesis if only a small amount of enzyme was applied (test 1 in Figure 4B). A surplus of the enzyme, however, could cope with this crucial inhibition so enhancing its activity during synthesis and yielding a high rate of product formation (Figures 4B and 5, test 2). An increase in sucrose synthase concentration lowered the level of dTDP and the accompanying dehydratase inhibition, leading to a further enlarged product formation with quantitative conversion after 6 h (Figure 5, test 3).

Preparative combined synthesis as a fed batch

With our two-enzyme system we completely converted the reactant dTDP to 1.3 g of dTDP-6-deoxy-4-keto-D-glucose (Figure 6). Striving for a high product concentration that generally simplifies the isolation procedure, the reaction was conducted as a fed batch in view of the nucleoside diphosphate. Thus, we limited the consequences of substrate inhibition of sucrose synthase, as well as the inhibition of the dehydratase by dTDP, yielding a final concentration of 44.2 mM (theoretical yield = 94%). The minor degradation of 6% of the nucleotide deoxysugar was caused by its inherent lability and some enzymatic hydrolysis resulting

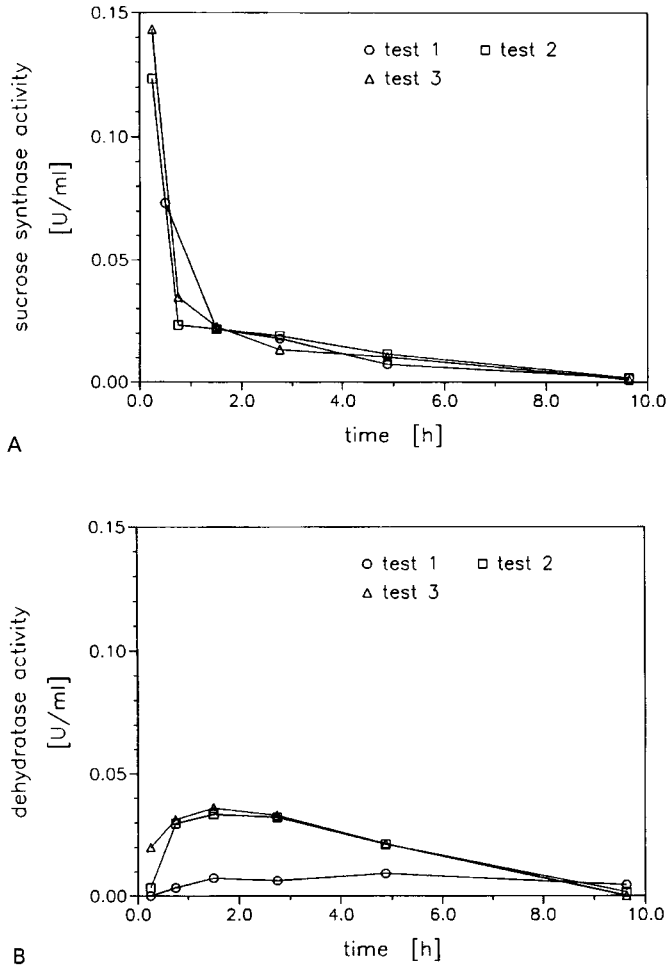

Figure 4. Enzyme activities under synthesis conditions. (A) activity of sucrose synthase. (B) activity of dTDP-D-glucose 4,6-dehydratase

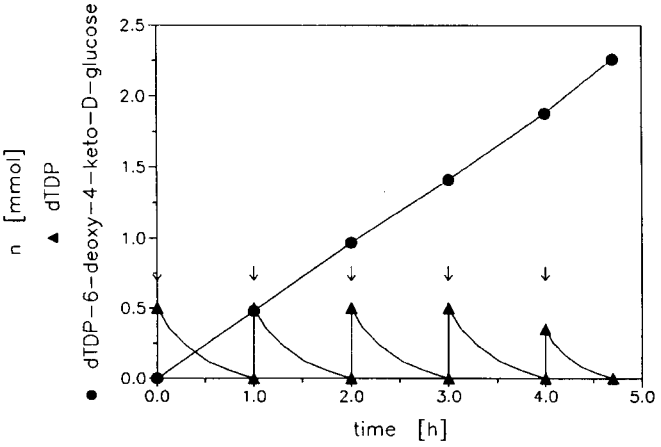

Figure 6. Fed batch synthesis: time course of dTDP and dTDP-6-deoxy-4-keto-D-glucose concentration. Arrows mark a feed of dTDP in 1 ml buffer.

Table 4. Isolation of dTDP-6-deoxy-4-keto-D-glucose.

Operation	*Yield [%]*
Fed batch synthesis	94
Deproteinization	95
Anion-exchange	86
Desalting	95
Lyophilization	100
Overall	73

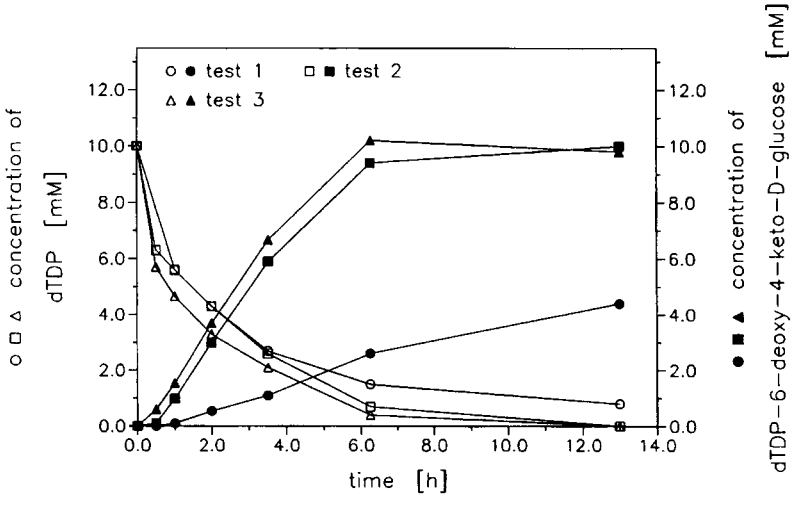

Figure 5. Conversion rates with different enzyme amounts.

from the sucrose synthase preparation. The space-time-yield of 133 $gl^{-1} d^{-1}$ (referred to the sodium salt with $M_r = 590.3$) is a good productivity, compared to literature data [25, 34, 35]. The enzyme consumption for a calculated 1 kg of product would be < 1100 U of sucrose synthase and 5900 U of the dehydratase. This demonstrates that a properly operated discontinuous process allows the same low catalyst costs as a continuous process [11]. In the present synthesis we replaced the extremely expensive dTDP-D-glucose for the nucleoside diphosphate which is cheaper by a factor of 2.5. The breakthrough concerning reactant costs was accomplished with our synthesis of NDP activated sugars (N = A, C, U, dU) starting from nucleoside monophosphates [36], these compounds being 14–32 times cheaper than the respective diphosphates. Work is in progress in our laboratory to realize the combined synthesis of dTDP-6-deoxy-4-keto-D-glucose starting from dTMP and sucrose.

Isolation of dTDP-6-deoxy-4-keto-D-glucose

The efficient three-step purification yielded 1.1 g of the final product (Table 4). The combined synthesis facilitated the isolation considerably because contaminants from commercial dTDP-D-glucose [9] were no longer present. In the anion-exchange chromatography dTMP (an impurity of commercial dTDP) was separated and the product eluted at 0.45 M sodium chloride. According to Marumo *et al.* [8] we also found that complete desalting was the absolute prerequisite to obtain undecomposed dTDP-6-deoxy-4-keto-D-glucose which was in a stable state from lyophilization. By NMR spectroscopy and ESI-MS [9] the deoxysugar was unequivocally characterized as pure compound, forming a 1:5 mixture of ketoform and its hydrate.

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