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One-pot Enzymatic Production of dTDP-4-keto-6-deoxy-D-glucose from dTMP and Glucose-1-phosphate

Jongmin Oh,[1] Sun-Gu Lee,[1] Byung-Gee Kim,[1] Jae Kyung Sohng,[2] Kwangkyoung Liou,[2] Hei Chan Lee[2]

[1]Institute of Molecular Biology and Genetics and School of Chemical Engineering, Seoul National University, Seoul, 151-742, South Korea; telephone: +82 2 880-6774; fax: +82 2 874-1206; e-mail: byungkim@snu.ac.kr
[2]Institute of Biomolecule Reconstruction Lab, Sunmoon University, Asan, 336-840, South Korea

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Abstract: An enzymatic production method for dTDP-4-keto-6-deoxy-D-glucose, a key intermediate of various deoxysugars in antibiotics, was developed starting from dTMP, acetyl phosphate, and glucose-1-phosphate. Four enzymes, i.e., TMP kinase, acetate kinase, dTDP-glucose synthase, and dTDP-D-glucose 4,6-dehydratase' were overexpressed using T7 promoter system in the *E. coli* BL21 strain, and the dTDP-4-keto-6-deoxy-D-glucose was synthesized by using the enzyme extracts in one-pot batch system. When 20 m*M* dTMP of initial concentration was used, Mg^{2+} ion, acetyl phosphate, and glucose-1-phosphate concentrations were optimized. About 95% conversion yield of dTDP-4-keto-6-deoxy-D-glucose was obtained based on initial dTMP concentration at 20 m*M* dTMP, 1 m*M* ATP, 60 m*M* acetyl phosphate, 80 m*M* glucose-1-phosphate, and 20 m*M* $MgCl_2$. The rate-limiting step in this multiple enzyme reaction system was the dTDP-glucose synthase reaction. Using the reaction scheme, about 1 gram of purified dTDP-4-keto-6-deoxy-D-glucose was obtained in an overall yield of 81% after two-step purification, i.e., anion exchange chromatography and gel filtration. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **84:** 452–458, 2003.

Keywords: dTDP-4-keto-6-deoxy-D-glucose; TMP kinase; acetate kinase; dTDP-glucose synthase; dTDP-D-glucose 4,6-dehydratase

INTRODUCTION

Despite the importance of dTDP-4-keto-6-deoxy-D-glucose (dTKDG) as a key intermediate for other dTDP-D- and L-6-deoxysugar derivatives (Amann et al. 2001; Stein et al. 1998), difficulty in the economical production of dTKDG has limited an efficient study on deoxysugar pathways. A typical synthetic method for dTKDG is using a two-enzyme system, i.e., dTDP-glucose synthase and dTDP-D-glucose 4,6-dehydratase, starting from glucose-1 phosphate and dTTP (Linton et al., 1995). This method is used by several groups to produce enough dTKDG to identify the subsequent reaction mechanisms of the synthesis of deoxysugar derivatives on a laboratory scale. In this process, the high cost of dTTP limits the large-scale production of dTKDG. To produce dTKDG economically, Stein et al. (1998) devised a combined reaction using sucrose synthase, which converts dTDP and sucrose into dTDP-glucose, and dTDP-D-glucose 4,6-dehydratase. This method is quite attractive since sucrose is a very cheap substrate. However, dTDP is more expensive than dTTP, and the reversible nature of the sucrose synthase reaction requires high concentration of sucrose to achieve high yield. Moreover, the sucrose synthase should be purified from rice.

Here, we suggest an economical enzymatic production method of dTKDG starting from dTMP, which is a much cheaper substrate compared to dTDP and dTTP (Fig. 1). In this reaction: (1) TMP-kinase is used for the conversion of dTMP to dTDP; (2) acetate kinase is for the ATP regeneration and for the conversion of dTDP to dTTP; (3) dTDP-glucose synthase is for the conversion of dTTP and glucose-1-phosphate to dTDP-glucose; (4) dTDP-D-glucose 4,6-dehydratase is for the conversion of dTDP-glucose to dTKDG. All the enzymes were overexpressed in *E. coli* and employed as extract forms for the reaction. The whole reaction was performed in one-pot. This work shows an efficient gram-scale synthesis of the dTKDG, which can be

Correspondence to: Byung-Gee Kim

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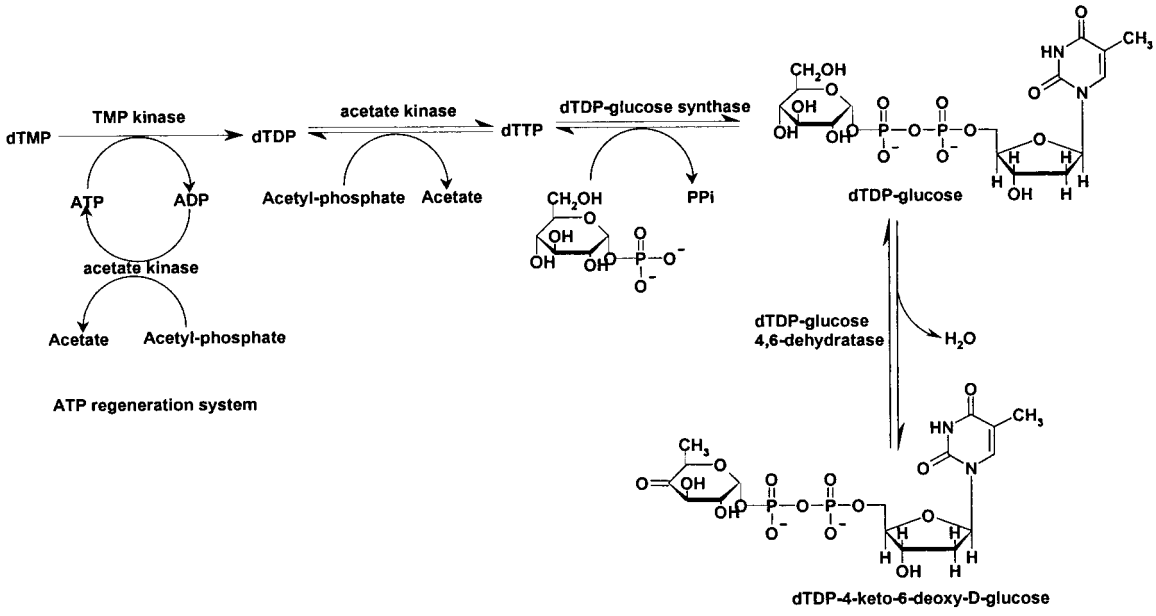

Figure 1. Schematic diagram of the production system for dTDP-4-keto-6-deoxy-D-glucose.

used for further synthesis of various deoxysugar units for antibiotics.

MATERIALS AND METHODS

Materials

All chemicals used in this study were purchased from Sigma (St. Louis, MO). The enzymes for DNA manipulations were from Boehringer Mannheim GmbH (Mannheim, Germany). pGEM-T easy vector system was from Promega Corporation (Madison, WI). pET15b and *E. coli* BL21(DE3) were from Novagen (Madison, WI), and pET24-ma was kindly donated by Dr. Hiroshi Sakamoto (Pasteur Institute, Paris, France).

Plasmids Construction

DNA manipulations were performed according to the procedures described by Sambrook et al. (1989) and the pGEM-T easy vector system was used for the cloning of PCR products. The TMP kinase (E.C.2.7.4.9) gene was amplified by PCR using the genomic DNA of *E. coli* K12 as template, and cloned into the plasmid pET15b containing the pBR322 replication origin and ampicillin (Am) resistance gene. The acetate kinase (E.C.2.7.2.1) and dTDP-glucose synthase (E.C.2.7.7.24) genes from *E. coli* K12 were cloned into the plasmid pET24ma containing the p15A origin and kanamycin (Km) resistance gene. The dTDP-D-glucose 4,6-dehydratase (E.C.4.2.1.46) from *Salmonella enterica serovar typhimurium LT2* was cloned in the pRSET-B from Invitrogen (Carlsbad, CA).

Expression of Enzymes

All the plasmids above were transformed into *E. coli* BL21 (DE3) and the cells were aerobically grown in 50 mL of Luria-Bertani (LB) medium using 250-mL shake flasks. For the selection pressure of each plasmid, 100 μg/mL of ampicillin or/and 50 μg/mL of kanamycin were used accordingly. The cells expressing TMP kinase or acetate kinase were grown to OD_{600} of 0.6 at 37°C, and then 1 m*M* isopropyl β-d-thiogalactopyranoside (IPTG) was used for the induction. The cells were harvested and pelleted after 6 h of the culture. Owing to the heavy inclusion body formation, dTDP-glucose synthase gene followed a somewhat different induction strategy. The *E. coli* with dTDP-glucose synthase gene was grown up to OD_{600}, of 0.6 at 27°C, and then 0.4 m*M* IPTG was added to induce the cloned gene. The cells were harvested and pelleted after 12 h of the culture. The *E. coli* with dTDP-D-glucose 4,6-dehydratase gene was cultured to OD_{600}, of 0.6 at 30°C, and 0.4 m*M* IPTG was used for the induction. The cells were harvested and pelleted after 10 h of the culture. Crude extracts were prepared by the sonication of the cell suspension in 50 m*M* Tris-HCl buffer (pH 7.5), containing 1 m*M* DTT, 1 m*M* PMSF, 1 m*M* EDTA, 1 m*M* $MgCl_2$. The lysate was then centrifuged at 15000*g* for 30 min at 4°C, and the precipitate was discarded.

Enzyme and Product Assay

All the enzyme activities were determined in 50 m*M* Tris-HCl buffer (pH 7.5) at 37°C. One unit of the enzyme activity was defined as the amount catalyzing the formation of 1 μmol product per min. TMP kinase activity was assayed

using 5 m*M* dTMP, 5 m*M* ATP, and 20 m*M* $MgCl_2$. Acetate kinase activity was assayed using 5 m*M* dTDP, 20 m*M* acetyl phosphate, and 20 m*M* $MgCl_2$. dTDP-glucose synthase activity was assayed using 5 m*M* dTTP, 20 m*M* glucose-1-phosphate, and 20 m*M* $MgCl_2$. dTDP-D-glucose 4,6-dehydratase was assayed using 5 m*M* dTDP-glucose. Various nucleotides were analyzed by HPLC using a strong anion exchange analytical column (SAX, 4.6 × 250 mm, 5 μm particle size; Alltech Associates (Deerfield, Illinois) with a linear gradient from 50 to 500 m*M* potassium phosphate buffer (pH 3.5) (Tomiya et al., 2001). The eluted nucleotides were monitored by absorbance at 270 nm. A Bruker Biflex III MALDI-TOF mass spectrometer (Bremen, Germany) and NMR spectrometer (Bruker Avance 500) were used for the product identification.

Enzyme Reactions for dTDP-4-keto-6-deoxy-D-glucose

The reaction for dTKDG was performed at pH 7.5 using 100 μL of 50 m*M* Tris buffer containing dTMP, ATP, acetyl phosphate, glucose-1-phosphate, $MgCl_2$, and cell extract at 37°C. (Detailed reaction conditions are described in the figure captions.) The reaction was stopped by heating the reaction mixture at 100°C for 30 s. The reaction mixture was diluted with the same buffer (1:10), and the reactions were monitored by HPLC analysis.

Preparative Synthesis of dTDP-4-keto-6-deoxy-D-glucose and Its Purification

For the synthesis of the gram scale of the product, the synthesis reaction was carried out in 100 mL reaction volume using a stirred 500-mL round flask. The initial concentrations of dTMP, ATP, acetyl phosphate, glucose-1-phosphate, and $MgCl_2$ were 20 m*M*, 1 m*M*, 60 m*M*, 80 m*M*, and 20 m*M*, respectively. Seventeen units of the three enzyme extracts and 6000 units of acetate kinase were added. The reaction was carried out for 80 min at 37°C. The dTKDG, was purified using anion exchange chromatography (Dowex 1 × 2, Cl^-) and gel filtration column (Sephadex G-15) (Stein et al., 1998). The product was characterized by ^{1}H-NMR, ^{13}C-NMR, and MALDI-TOF and its purity was analyzed by HPLC.

RESULTS

Expression of Recombinant Enzymes

The four enzymes, i.e., TMP kinase, acetate kinase, dTDP-glucose synthase, and dTDP-D-glucose 4,6-dehydratase, were over-expressed under T7 promoter system in *E.coli* BL21 (DE3) by IPTG induction. Their expressions and activities were confirmed by 12% SDS-PAGE and enzyme assay (data not shown). TMP kinase and acetate kinase were expressed as their soluble forms at normal induction condition, i.e., 1 m*M* IPTG and 37°C. However, most of the expressed dTDP-glucose synthase was insoluble at normal induction condition (data not shown). Thus, the optimized condition was determined by varying culture temperature, induction OD, and IPTG concentration. The expression of even the slight soluble form of dTDP-glucose synthase was possible only at below 27°C. The expression of dTDP-D-glucose 4,6-dehydratase was not successful at 37°C for unknown reasons, but was good at below 30°C. A possible reason would be a high intracellular degradation rate of the enzyme by proteolysis at 37°C. The optimized condition for each enzyme was described in the Materials and Methods section. The obtained enzyme activities of TMP kinase, acetate kinase, dTDP-glucose synthase, and dTDP-D-glucose 4,6-dehydratase in the crude extracts were 19.6, 10970, 2.5, and 3.9 units/mg, respectively. Cell extracts contained very low contaminating phosphatase activity and could be used successfully for enzymatic synthesis.

Effect of Mg^{2+}, Acetyl Phosphate, and Glucose-1-phosphate on the Production of dTKDG

As four enzymes are involved in the reaction shown in the Figure 1, reaction parameters such as pH and substrate concentration would affect each enzyme differently. To determine the optimum condition of the whole reaction for dTKDG, we examined the effect of Mg^{2+}, acetyl phosphate, and glucose-1-phosphate on the production of dTKDG. In this experiment, the pH was set to 7.5 considering the optimized reaction pH for each enzyme (TMP kinase: 7.5, acetate kinase: 7.5, dTDP-glucose synthase: 8.0: dTDP-D-glucose 4,6-dehydratase: 7.5). Initial dTMP concentration was fixed at 20 m*M* and 1 m*M* ATP was used considering the Km value of *E. coli* acetate kinase for ADP, i.e., 0.5 m*M* (Fox and Roseman, 1986; Hirschbein et al., 1982). All reactions reached their steady state after 2 h, and the data in Figure 2 show the conversion yield of dTKDG based on the initial [dTMP] at the time.

Kinase and synthase activities are greatly affected by the level of divalent cations and ATP level. According to the previous reports, *E. coli* TMP kinase shows its highest activity by the activation of Mg^{2+} (Reynes et al., 1996), and *E. coli* acetate kinase shows optimum activity at Mg^{2+} to ATP (or ADP) ratio of 1.5 : 1 to 2 : 1 (Fox and Roseman, 1986). dTDP-glucose synthase from *E. coli* shows an optimum activity at Mg^{2+} to ATP (or ADP) ratio of 2.5 : 1 (Amann et al., 2001). Unlike other enzymes, dTDP-D-glucose 4,6-dehydratase from *Salmonella* is neither activated nor inhibited by Mg^{2+} ion (data not shown). To determine the required optimal Mg^{2+} concentration for the whole dTKDG production process, we examined the production rate of dTKDG by varying the $MgCl_2$ concentration from 0 m*M* to 60 m*M*. $MgCl_2$ concentration is almost linearly proportional to the conversion yield of dTKDG up to 20 m*M*, but the yield was not improved beyond 20 m*M* of $MgCl_2$ (Fig. 2a).

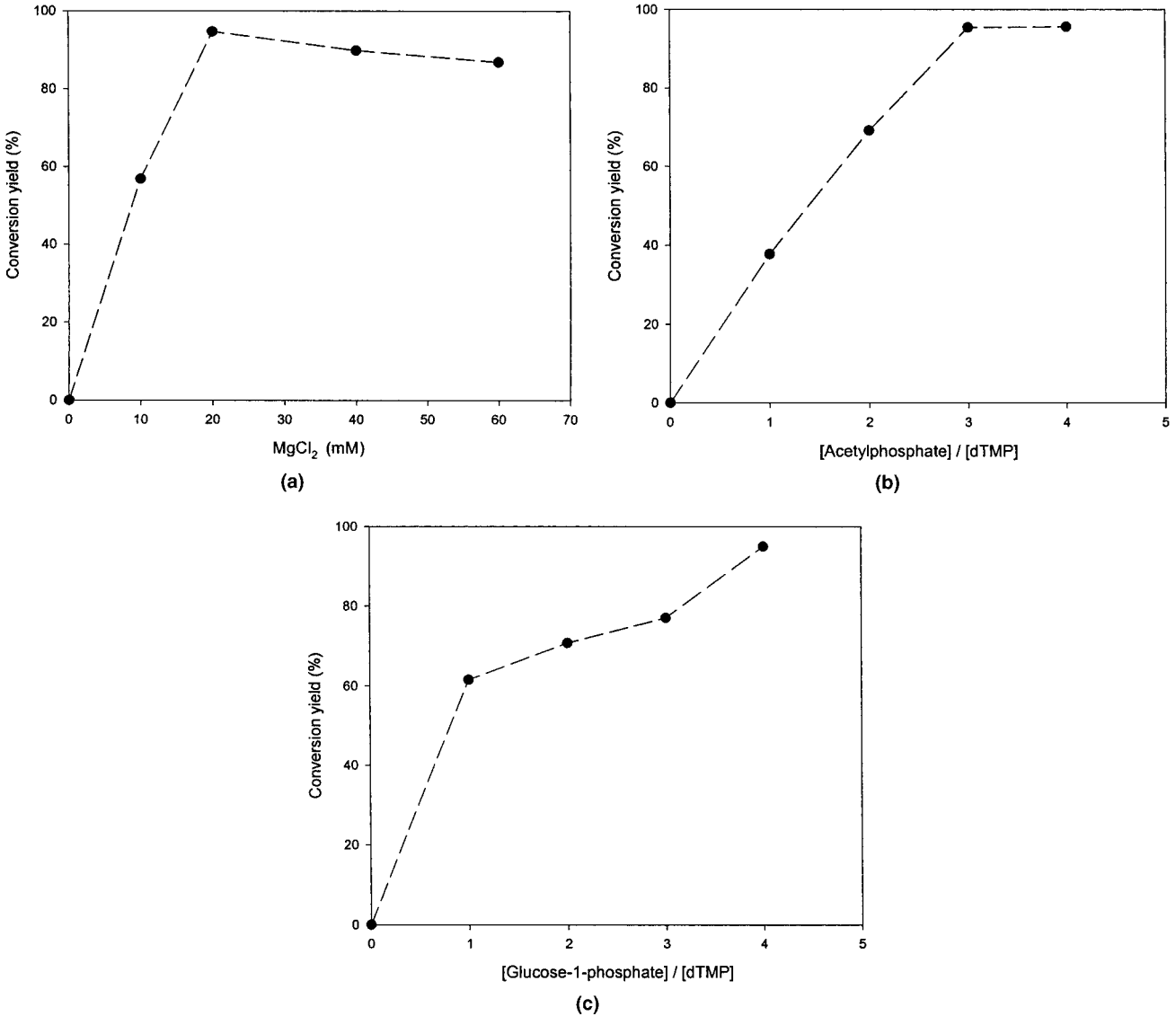

Figure 2. Effect of the concentrations of Mg^{2+}, acetyl phosphate, and glucose-1-phosphate on the production of dTKDG. All reactions were performed with 20 m*M* dTMP, 1 m*M* ATP, and 0.17 units of TMP kinase, TDP-glucose synthase, dTKDG dehydratase, and 60 units of acetate kinase in 100 μL of Tris buffer (pH 7.5). Other reaction conditions were described separately. (a) The effect of $MgCl_2$ concentration on the dTKDG production. Reaction condition: 80 m*M* glucose-1-phosphate, 80 m*M* acetyl phosphate, 0, 10, 20, 40, and 60 m*M* $MgCl_2$. (b) The effect of [acetyl phosphate]/[dTMP] on the production of dTKDG. Reaction condition: 80 m*M* glucose-1-phosphate, 20 m*M* $MgCl_2$, 0, 20, 40, 60, and 80 m*M* acetyl phosphate. (c) The effect of [glucose-1-phosphate]/[dTMP] on the production of dTKDG. Reaction condition: 0, 20, 40, 60, and 80 m*M* glucose-1-phosphate, 80 m*M* acetyl phosphate, 20 m*M* $MgCl_2$.

The concentration of acetyl phosphate, a phosphate donor for the conversion of dTMP to dTTP and ADP to ATP, was varied from 0 m*M*–80 m*M*. Theoretically, if an equivalent amount of ATP or dTTP is generated from one acetyl phosphate molecule, two equivalent moles of acetyl phosphate are required for the complete conversion of dTMP into dTTP. However, the conversion yield of dTKDG becomes maximum at [acetyl phosphate]/[dTMP] ratio of 3 (Fig. 2b). At the [acetyl phosphate]/[dTMP] ratio of 2, only 70% of the conversion yield was achieved based upon the amount of dTMP batched. This would be mainly caused by the instability of acetyl phosphate, as the half-life of acetyl phosphate in aqueous solution is about 8 h (Hirschbein et al., 1982). Thus, it is desirable that the acetyl phosphate is added in excess in the beginning of the reaction or continuously added for the efficient conversion of dTMP.

Likewise the concentration of glucose-1-phosphate, i.e., glucose donor for the conversion of dTTP to dTDP-glucose, was varied from 0 m*M*–80 m*M*. Until the glucose-1-phosphate concentration is 4 times higher than the dTMP concentration, the conversion yield of dTKDG increased up to 95% (Fig. 2c), suggesting that dTDP-glucose synthesis reaction is a reversible reaction and the equilibrium

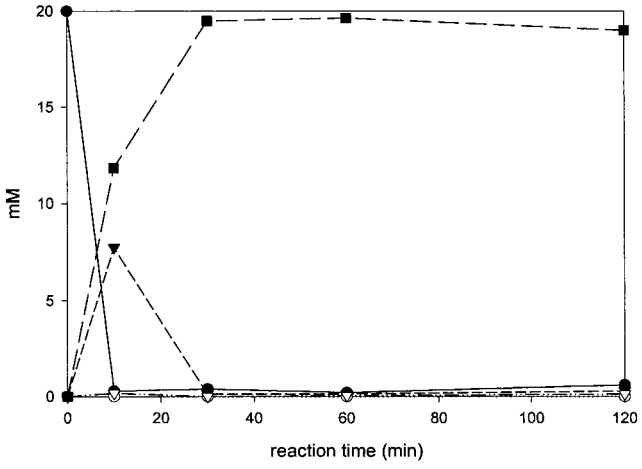

Figure 3. Reaction profile of dTKDG production using four enzyme extracts. Reaction condition: 20 m*M* dTMP, 1 m*M* ATP, 60 m*M* acetyl phosphate, 80 m*M* glucose-1-phosphate, 20 m*M* $MgCl_2$, 0.17 units of TMP kinase, TDP-glucose synthase, dTKDG dehydratase and 60 units of acetate kinase in 100 μL of Tris buffer (pH 7.5). (●) dTMP, (○) dTDP, (▼) dTTP, (▽) dTDP-glucose, (■) dTDP-4-keto-6-deoxy-glucose.

constant appears to be lower than the other two kinases. The instability of glucose-1-phosphate in the reaction buffer would also be a possibility.

Gram-Scale Synthesis of dTDP-4-keto-6-deoxy-D-glucose

First, the production of dTKDG was investigated in a small-scale batch system (100 μL) using the optimized condition starting from dTMP and glucose-1-phosphate. The synthesis reaction was initiated by the addition of 0.17 units of the three enzymes and 60 units of acetate kinase. The acetate kinase was used in excess for efficient ATP regeneration and preventing accumulation of dTDP, an inhibitor of dTDP-D-glucose 4,6-dehydratase (Stein et al., 1998). Figure 3 displays the profiles of dTMP, dTDP, dTTP, dTDP-glucose, and dTKDG. The conversion yield of dTKDG based on the initial dTMP concentration reached about 95% in only 30 min. The reaction profiles suggest that the overall rate-limiting step in the synthesis of dTKDG would be dTDP-glucose synthesis, because dTTP accumulates within 10 min and gradually decreases afterwards. During the reaction, dTDP and dTDP-glucose were not nearly detected. This result indicates that the inhibition of the dTDP-D-glucose 4,6-dehydratase by dTDP would not be a problem.

The same optimized reaction condition was applied to a gram-scale synthesis in 100 mL reaction volume. Compared to the 100 μL scale reaction, only 100-fold increased amounts of the enzymes were used, as the amounts of enzymes required to completely proceed the same reaction within several hours are sufficient with one tenth of the levels used for the 100 μL scale reaction. As the amounts of the used enzymes are sufficient enough to convert the substrates into products within 30 min, the reaction profile was still quite similar to that of the 100 μL scale reaction (data not shown). The final conversion yield of dTKDG based on initial dTMP was about 97% (1.148 g). This result indicates that dTKDG can be very efficiently produced in gram scale from relatively cheap substrate, i.e., dTMP and glucose-1-phosphate.

After the completion of the reaction for dTKDG, the product was purified by protein removal, anion exchange

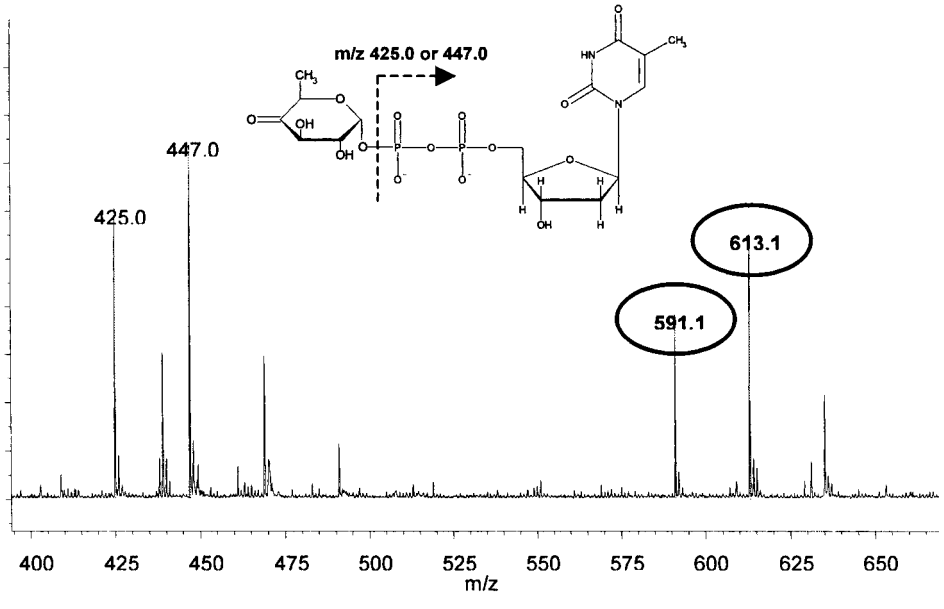

Figure 4. MALDI-TOF (positive-ion mode) spectrum of dTDP-4-keto-6-deoxy-D-glucose. The MALDI-TOF equipped with a pulsed 337 nm nitrogen laser was used at 400 μJ/pulse energy level. 0.5 μL of the sample and 0.5 μL of the matrix solution (0.5*M* dihydroxybenzoic acid in methanol) were mixed on the target plate. Spectrums of 100 shots were accumulated. The spectra were externally calibrated with bradkynin (MH+ = 905.0440) and alanine (MH+ = 90.1020).

column, and desalting. The desalted fraction was lyophilized and 0.96 g of dTKDG white powder was obtained. Overall yield of the product was 81% based on initial dTMP and the purity was approximately 96%.

The positive-ion mode of MALDI-TOF spectrum for purified dTKDG showed two intense peaks at m/z 591.1 and 613.1 corresponding to the [M+2Na-H] and [M+3Na-2H], and the other two peaks at m/z 425.0 and 447.0 corresponding to the [M+2Na-H] and [M+3Na-2H]. These peaks at low mass region agree well with the decomposed dTDP (Fig. 4). The purified product was also identified with 500 MHz ^{1}H-NMR and 500 MHz ^{13}C-NMR (data not shown), which agreed well with previous findings (Amann et al., 2001; Naundorf and Klaffke, 1996).

DISCUSSION

In this article, we have shown that the key intermediate of most deoxysugars in antibiotics, i.e., dTDP-4-keto-6-deoxy-D-glucose, can be efficiently produced from dTMP using four recombinant enzyme extracts in one-pot. The advantage of this process is that the dTKDG can be produced from relatively cheap substrate, dTMP and glucose-1-phosphate. dTMP is approximately 20–30 times cheaper than dTDP and dTTP, and it would be possible to provide glucose1-phospate from glucose at the end in the near future. Many enzymatic processes have been suggested for the efficient production of nucleotide-sugars such as UDP-galactose, CMP-NeuAc, and GDP-mannose from NMP (Butler and Elling, 1999). In those processes, adenylate kinase was used for the conversion of each NMP to NDP. However, adenylate kinase does not show any substrate specificity for dTMP, resulting in the difficulty in the use of dTMP for dTDP-sugar production (Zervosen et al., 1996). We have overcome this problem by employing the recombinant TMP-kinase for the conversion of dTMP to dTDP. To our knowledge, the production of dTKDG reported here is the first example of the production of dTDP-sugar from dTMP.

Although acetate kinase was employed for the ATP regeneration and the conversion of dTDP to dTTP in our reaction scheme, pyruvate kinase would be an alternative enzyme to execute the reactions (Hirchbein et al., 1982). Since phospho(enol)pyruvate, a phosphate donor for pyruvate kinase, is very stable compared to acetyl phosphate, pyruvate kinase system has been also popularly employed for the production of sugar-nucleotides (Butler and Elling, 1999). We could confirm that dTKDG was also produced by using the pyruvate kinase in our system. Nevertheless, we employed the acetate kinase system in our process for the following reasons. First, when we compared the two systems using the same units of enzymes and the same concentrations of phosphate donors, the reaction efficiency using pyruvate kinase was slightly lower compared to the system using acetate kinase (data not shown). Second, the phospho(enol)pyruvate is about 5 times more expensive than acetyl phosphate.

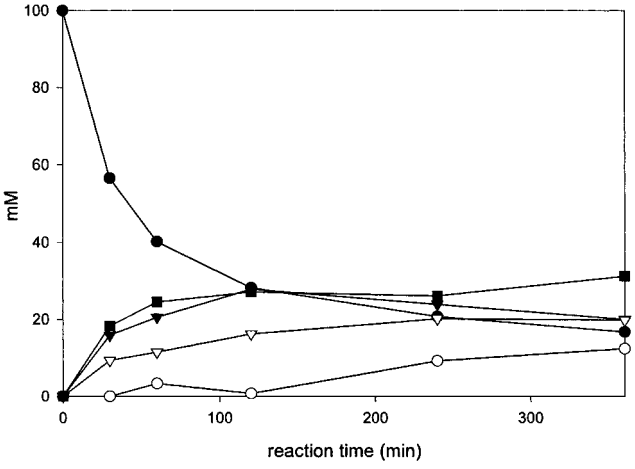

Figure 5. Reaction profile of dTKDG production using high substrate concentration. Reaction condition: 100 m*M* dTMP, 5 m*M* ATP, 300 m*M* acetyl phosphate, 400 m*M* glucose-1-phosphate, 100 m*M* $MgCl_2$, 0.34 units of TMP kinase, TDP-glucose synthase, dTKDG dehydratase, and 120 units of acetate kinase in 100 μL of Tris buffer (pH 7.5). (●) dTMP, (○) dTDP, (▼) dTTP, (▽) dTDP-glucose, (■) dTDP-4-keto-6-deoxy-glucose.

In our experiments, we used 20 m*M* dTMP as an initial concentration for the production of dTKDG. However, in the aspect of large-scale production, the use of high substrate concentration would be more advantageous. We tried to perform the same reaction using 100 m*M* dTMP as an initial concentration. The concentrations of glucose-1-phosphate, ATP, and acetyl phosphate were increased with the same scale. However, the conversion yield of dTKGD was only about 30% based on initial TMP concentration (Fig. 5). This would be caused by the inhibition of enzymes by employing high concentrated reactants. In addition, we could observe the precipitation of acetyl phosphate complexed with magnesium ion, which would also cause the low conversion yield. Fed-batch type addition of the substrates would overcome this kind of problem.

A major problem in performing our process for dTKDG would be the availability of dTDP-glucose synthase. Even though we could obtain the enzyme activity sufficient to conduct our experiments by optimizing the expression condition, the formation of the inclusion body was still a major portion of the enzyme. To overcome this problem, we are trying to refold the inclusion body and use soluble fusion proteins.

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