

12-12-2005

Preparative Synthesis of dTDP-L-Rhamnose Through Combined Enzymatic Pathways

Young Bok Abraham Kang
George Fox University, ykang@georgefox.edu

Yung-Hun Yang
Seoul National University

Kwang-Won Lee
Seoul National University

Sun-Gu Lee
Pusan National University

Jae Kyung Sohng
Sunmoon University

See next page for additional authors

Follow this and additional works at: https://digitalcommons.georgefox.edu/mece_fac

 Part of the [Biomedical Engineering and Bioengineering Commons](#)

Recommended Citation

Kang, Young Bok Abraham; Yang, Yung-Hun; Lee, Kwang-Won; Lee, Sun-Gu; Sohng, Jae Kyung; Lee, Hei Chan; Liou, Kwangkyoung; and Kim, Byung-Gee, "Preparative Synthesis of dTDP-L-Rhamnose Through Combined Enzymatic Pathways" (2005). *Faculty Publications - Biomedical, Mechanical, and Civil Engineering*. 62.
https://digitalcommons.georgefox.edu/mece_fac/62

This Article is brought to you for free and open access by the Department of Mechanical and Civil Engineering at Digital Commons @ George Fox University. It has been accepted for inclusion in Faculty Publications - Biomedical, Mechanical, and Civil Engineering by an authorized administrator of Digital Commons @ George Fox University. For more information, please contact arolfe@georgefox.edu.

Authors

Young Bok Abraham Kang, Yung-Hun Yang, Kwang-Won Lee, Sun-Gu Lee, Jae Kyung Sohng, Hei Chan Lee, Kwangkyoung Liou, and Byung-Gee Kim

Preparative Synthesis of dTDP-L-Rhamnose Through Combined Enzymatic Pathways

Young-Bok Kang,¹ Yung-Hun Yang,¹ Kwang-Won Lee,² Sun-Gu Lee,³ Jae Kyung Sohng,⁴ Hei Chan Lee,⁴ Kwangkyoung Liou,⁴ Byung-Gee Kim^{1,2}

¹School of Chemical and Biological Engineering, Seoul National University, Seoul, 151-742, Korea; telephone: +82-2-880-6774; fax: +82-2-874-1206;

e-mail: byungkim@snu.ac.kr

²Interdisciplinary Program for Biochemical Engineering and Biotechnology, Seoul National University, Seoul, 151-742, Korea

³Department of Chemical and Biochemical Engineering, Pusan National University, Busan, 609-735, Korea

⁴Institute of Biomolecule Reconstruction, Sunmoon University, Asan, 336-840, Korea

Abstract: dTDP-L-rhamnose, an important precursor of O-antigen, was prepared on a large scale from dTMP by executing an one-pot reaction in which six enzymes are involved. Two enzymes, dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase, responsible for the conversion of dTDP-4-keto-6-deoxy-D-glucose to dTDP-L-rhamnose, were isolated from their putative sequences in the genome of *Mesorhizobium loti*, functionally expressed in *Escherichia coli*, and their enzymatic activities were identified. The two enzymes were combined with an enzymatic process for dTDP-4-keto-6-deoxy-D-glucose involving TMP kinase, acetate kinase, dTDP-glucose synthase, and dTDP-glucose 4,6-dehydratase, which allowed us to achieve a preparative scale synthesis of dTDP-L-rhamnose using dTMP and glucose-1-phosphate as starting materials. About 82% yield of dTDP-L-rhamnose was obtained based on initial dTMP concentration at 20 mM dTMP, 1 mM ATP, 10 mM NADH, 60 mM acetyl phosphate, and 80 mM glucose-1-phosphate. From the reaction with 20 ml volume, approximately 180 mg of dTDP-L-rhamnose was obtained in an overall yield of 60% after two-step purification, that is, anion exchange chromatography and gel filtration for desalting. The purified product was identified by HPLC, ESI-MS, and NMR, showing about 95% purity.

© 2005 Wiley Periodicals, Inc.

Keywords: dTDP-L-rhamnose; dTDP-4-keto-6-deoxy-D-glucose; dTDP-4-keto-6-deoxy-D-glucose 3,5 epimerase; dTDP-4-keto-rhamnose reductase

INTRODUCTION

dTDP-L-rhamnose is a precursor of L-rhamnose, a representative deoxysugar found in O-antigen-specific oligosaccharides, polyketides, and antibiotics such as streptomycin and novobiocin (Varki, 1993). Owing to the crucial biological

activities of L-rhamnose, a large amount of the sugar-nucleotide is necessary to conduct many related studies such as the inhibitory mechanism of the biosynthetic pathways for L-rhamnose and the effect of carbohydrate modification on antibiotics (Amann et al., 2001; Steffensky et al., 2000). However, chemical synthesis of dTDP-L-rhamnose is very difficult, and an efficient enzymatic synthesis process has not been established for the sugar-nucleotide. Although an enzymatic method from dTDP-glucose using the rfb gene cluster of *salmonella enterica* LT2 was suggested (Marumo et al., 1992), the process is impractical to be used industrially because dTDP-glucose is still a very expensive substrate.

Multiple enzymatic processes have been developed to produce various sugar-nucleotides from cheap and easily available substrates based on the generation of efficient and economical pathways for target compounds following de novo (or *le Loir*) pathways of carbohydrates synthesis (Bulter and Elling, 1999). Until now, most developed processes have utilized nucleoside monophosphates (NMPs) as starting substrates due to their relatively low cost. For instance, UDP-galactose, GDP-fucose, and CMP-sialic acid could be efficiently produced from UMP, GMP, and CMP with their corresponding sugars by incorporating a few enzymatic steps (Bulter and Elling, 1999; Lee et al., 2002). In this study, we show that dTDP-L-rhamnose can be efficiently produced from cheap substrate dTMP using 6 enzyme steps involved in the biosynthetic pathway (Fig. 1). In our previous report, we have suggested the synthesis of dTDP-4-keto-6-deoxy-D-glucose, a key intermediate of various deoxysugars, from dTMP using dTMP kinase, acetate kinase, dTDP-glucose synthase, and dTDP-glucose 4,6-dehydratase (Oh et al., 2003). Here we extended the pathway to produce dTDP-L-rhamnose starting from dTMP by additionally incorporating dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase.

One of the critical points in the success of constructing economical enzymatic pathways is the overproduction of

Correspondence to: Byung-Gee Kim

Contract grant sponsors: Ministry of Commerce, Industry and Energy (MOCIE); Nano Bioelectronics & Systems Research Center (ERC) of Seoul National University

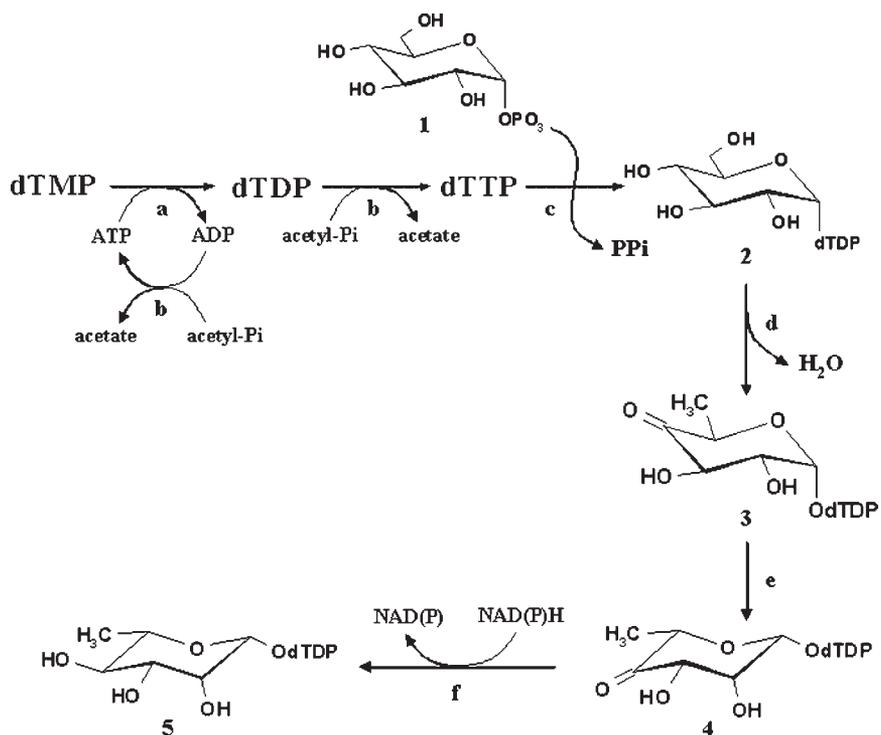


Figure 1. Schematic diagram of the biosynthesis of dTDP-L-rhamnose. (1) Glucose-1-phosphate, (2) dTDP-glucose, (3) dTDP-4-keto-6-D-glucose, (4) dTDP-4-keto-rhamnose, (5) dTDP-L-rhamnose. **a:** dTMP kinase, **(b)** acetate kinase, **(c)** dTDP-glucose synthase, **(d)** dTDP-glucose 4,6-dehydratase, **(e)** dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase, **(f)** dTDP-4-keto-rhamnose reductase.

functional enzymes required for the pathways. However, overexpression of recombinant protein in *E. coli* often forms nonfunctional inclusion bodies (Baneyx and Mujacic, 2004). In practice, when dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase from *E. coli* K12 strain were expressed to execute the reaction shown in the Figure 1, they could not be produced as soluble forms (unpublished data). Although many methods, such as refolding of protein, co-expression with chaperone and protein engineering, have been developed to overcome the problem of inclusion body formation, it is well known that they cannot be generally applied to all proteins (Baneyx and Mujacic, 2004). In this study, we attempted the screening of soluble and functional dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase from their corresponding putative genes in public microbial genomes. The putative genes of *Mesorhizobium loti* for the two enzyme were cloned, functionally overexpressed in *E. coli*, identified, and utilized for the production of dTDP-L-rhamnose, leading to the hundreds milligram scale production of dTDP-L-rhamnose.

MATERIALS AND METHODS

Materials

dTDP-4-keto-6-deoxy-D-glucose was prepared following the method reported elsewhere (Oh et al., 2003), and other chemical reagents used in this study were purchased from

Sigma (St. Louis, MO). *E. coli* BL21 (DE3) was from Novagen (West Palm Beach, FL), and pET24ma was kindly donated by Dr. Hiroshi Sakamoto (Pasteur Institute, Paris, France). pBAD and *E. coli* MC1061 were kindly donated by Prof. Deog Su Hwang (Seoul National University, Seoul, Korea).

Plasmid Construction

Two genes, that is, *mlr7511* and *mlr7533*, were amplified using the chromosomal DNA of *M. loti* as template and cloned into pET24ma and pBAD, respectively, to give pJMLR7551 and pJMLR7533. Following sets of primers were used for amplification: for the *mlr7551* gene, 5'-TTGGAGGGCGGATCCTTGGAGATCAGGTCCCTC-3' (creating a *Bam*HI site) and 5'-CTATCCTCAGAGCTCTCAGGCAAACACTTCGGC-3' (creating a *Sac*I site); for the *mlr7533* gene, 5'-AAGGTGCCATGGGGAGGCTCGCTGTCACCGG-3' (creating a *Nco*I site) and 5'-GTCATCCTCAGAGTCAGAACAGGACCCGACGC-3' (creating a *Xho*I site).

Expression and Preparation of Enzymes

pJMLR7551 and pJMLR7533 were transformed into *E. coli* BL21 (DE3) and *E. coli* MC1061, respectively. The cells were aerobically grown in 50 mL of Luria Bertani (LB) medium. Cells harboring pJMLR7551 gene was grown to OD₆₀₀ of 0.6 at 30°C, and then 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) was used for the induction.

The cells harboring pJMLR7553 gene was grown to OD₆₀₀ of 0.6 at 37°C, and then 1% arabinose (w/v) was used for the induction. The cells were harvested and pelleted after 12 h of the culture. Crude extracts were prepared by the sonication of the cell suspension, and dTMP kinase, acetate kinase, dTDP-glucose synthase, and dTDP-glucose 4,6-dehydratase were prepared as previously reported (Oh et al., 2003).

Enzyme Assays

The activities of dTDP-4-keto-6-deoxy-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase were identified by determining the net activity from dTDP-4-keto-6-deoxy-D-glucose to dTDP-L-rhamnose as reported elsewhere (Granger et al., 1999). A reaction mixture (pH 7.5, total volume: 100 µL) containing 0.5 µmol of dTDP-4-keto-6-deoxy-D-glucose, 0.5 µmol of NADH, 2 µmol of MgCl₂, 5 µmol of ammonium bicarbonate, and an appropriate amount of cell extracts was incubated at 37°C. The reaction was monitored by HPLC method as described below, and the net activity of dTDP-4-keto-6-deoxy-D-glucose and dTDP-4-keto-rhamnose reductase was expressed as enzyme unit (U), defined as the amount of enzyme catalyzing the formation of 1 µmol dTDP-L-rhamnose per min. The activities of dTMP kinase, acetate kinase, dTDP-glucose synthase, and dTDP-glucose 4,6-dehydratase were measured as previously reported (Oh et al., 2003).

Synthesis of dTDP-L-Rhamnose from dTMP and Glucose-1-Phosphate and Its Purification

The reaction for dTDP-L-rhamnose was performed at pH 7.5 using 100 µL of 50 mM ammonium bicarbonate buffer containing dTMP, glucose-1-phosphate, acetyl phosphate, NADH, MgCl₂, and cell extracts for six enzymes at 37°C (the detailed reaction condition was elaborated in the figure caption). Ten microliters of sample was withdrawn, diluted with the same buffer (1:10), and the reactions were analyzed by HPLC analysis.

For the preparative synthesis of dTDP-L-rhamnose, the synthesis reaction was carried out in 20 mL reaction volume. The initial concentrations of dTMP, ATP, acetyl phosphate, glucose-1-phosphate, NADH, and MgCl₂ were 20 mM, 1 mM, 60 mM, 80 mM, 10 mM, and 20 mM, respectively. After the complete conversion of dTMP to dTDP-L-rhamnose, the proteins were removed from the product solution by ultrafiltration. The product was purified using anion exchange (Dowex 1 × 2, Cl⁻) and gel filtration (Sephadex G-15) columns. The lyophilized product was characterized with ¹H-NMR, ¹H-COSY, ¹H-NOESY, ¹³C-NMR, and ESI-MS, and its purity was analyzed by HPLC.

Instrumental Analysis

Various nucleotide-sugars were analyzed by HPLC using an anion exchange analytical column (Carbopac PA1, 4.6 × 250 mm, 5 µm particle size, Dionex Associates, Netherlands)

with isocratic 200 mM ammonium dipotassium phosphate buffer (pH 7.5) at 1 mL/min. The eluted nucleotides were monitored by absorbance at 254 nm. Retention times at the condition were as follows: dTMP (3.1 min), dTDP (12.5 min), dTTP (90.0 min) dTDP-glucose(8.4 min), TDP-4-keto-6-deoxy-D-glucose(9.5 min), and dTDP-L-rhamnose (6.0 min).

ESI mass spectrometry analysis was performed in negative ion mode at a voltage of 5 kV with a Thermo LCQ ion trap mass spectrometer (Thermo Electron Corp, Waltham, MA) equipped with the standard Thermo ESI source. Samples were infused by syringe pump at a rate of 2 µL/min in 50% methanol and nebulized with dry nitrogen gas. The heated capillary was maintained at a temperature of 300 K. The maximum ion collection time was set at 50 ms, and five micro scans were summed per scan.

For the identification of final product, the purified products were dissolved in 700 µL D₂O (99.9 atom % D) and used for NMR analysis. ¹H, ¹H-COSY, ¹H-NOESY, and ¹³C-NMR spectra were recorded with a Bruker 500-MHz NMR spectrometer at a probe temperature of 298 K, and chemical shifts were expressed in ppm relative to internal HOD signal (4.80).

RESULTS

Identification of the Putative Genes for dTDP-4-Keto-6-Deoxy-D-Glucose 3, 5-Epimerase and dTDP-4-Keto-Rhamnose Reductase in *M. loti*

In our previous study, dTDP-4-keto-6-deoxy-D-glucose (dTKDG), an intermediate of dTDP-L-rhamnose, was successfully synthesized starting from dTMP by using four recombinant enzymes, that is, dTMP kinase, acetate kinase, dTDP-glucose synthase from *E. coli* and dTDP-glucose 4, 6-dehydratase from *Salmonella enterica* Serovar *Typhimurium* LT2 (Oh et al., 2003). To produce dTDP-L-rhamnose efficiently as an extended pathway from dTKDG, two additional enzymes, that is, dTDP-4-keto-6-deoxy-glucose 3, 5-epimerase and dTDP-4-keto-rhamnose reductase, are required to be combined with the four enzyme reactions for the synthesis of dTKDG. The two additional enzymes are found in some microorganisms containing rhamnose in LPS molecules, and known to catalyze the conversion of dTKDG to dTDP-L-rhamnose. First, we cloned the two enzymes from *E. coli* K12 and expressed under T7 promoter. However, the recombinant enzymes were not functional due to the formation of inclusion body (data not shown). To overcome this problem, we attempted to clone the two enzymes from different microorganisms whose genomic sequences were elucidated because proteins from different sources can show different solubility (Baneyx and Mujacic, 2004). To access the two enzymes, we searched genome database and could find out that two genes, that is, mlr7551 (GI 13476272) and mlr7553 (GI 13476274), in the genome of *M. loti* (GenBank accession number NC_002678) were annotated as putative genes for dTDP-4-keto-6-deoxy-glucose 3,5-epimerase

and dTDP-4-keto-rhamnose reductase, respectively (Kaneko et al., 2000).

To identify the function of the putative genes, we cloned and expressed them in *E. coli*. First, the two genes were cloned into pET system, and overexpressed under T7 promoter. When the expressed proteins were analyzed by SDS protein gel, we observed that Mlr7551 protein was a soluble form (Fig. 2), but most of Mlr7553 was insoluble (data not shown). To obtain the soluble form of Mlr7553 protein, the gene was cloned into pBAD vector controlled by araBAD promoter. The araBAD promoter is known to be very tightly regulated, therefore giving some window to control the soluble expression level of a protein (Haldimann et al., 1998). The optimum arabinose concentration was determined to be 1% (w/v), yielding soluble form of Mlr7553. Figure 2 shows the SDS-analysis of the soluble fractions and total fractions of proteins from mlr7551 and mlr7553 gene expressed using the pET and pBAD systems, respectively. The data indicate that more than 90% of the proteins were expressed as soluble forms, and the apparent molecular weights agreed with their theoretical ones (Mlr7551, 21 kDa; Mlr7553, 32 kDa) from their gene sizes (mlr7551, 552 bp; mlr7553, 879 bp).

To investigate the functional activities of the expressed proteins from mlr7551 and mlr7553, we monitored the formation of dTDP-L-rhamnose using ion-pair reversed-phase HPLC and ESI-MS after incubating dTKDG with the expressed enzymes. Figure 3A(a) shows the HPLC profile at

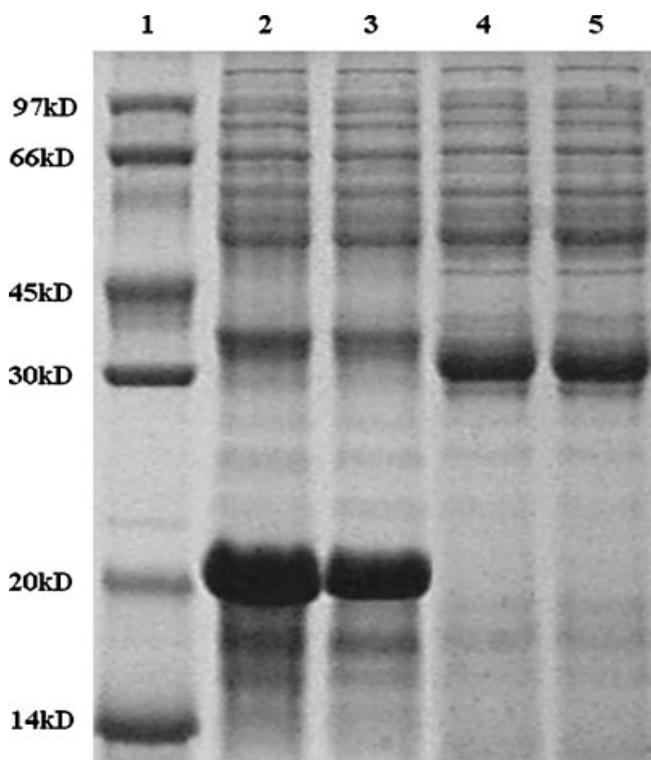


Figure 2. SDS-PAGE analysis of the induced proteins from the recombinant *E. coli* strains. **Lane 1:** Low molecular weight marker; (**lane 2**) soluble fraction for Mlr7551; (**lane 3**) total protein for Mlr7551; (**lane 4**) soluble fraction for Mlr7553; (**lane 5**) total protein for Mlr7553.

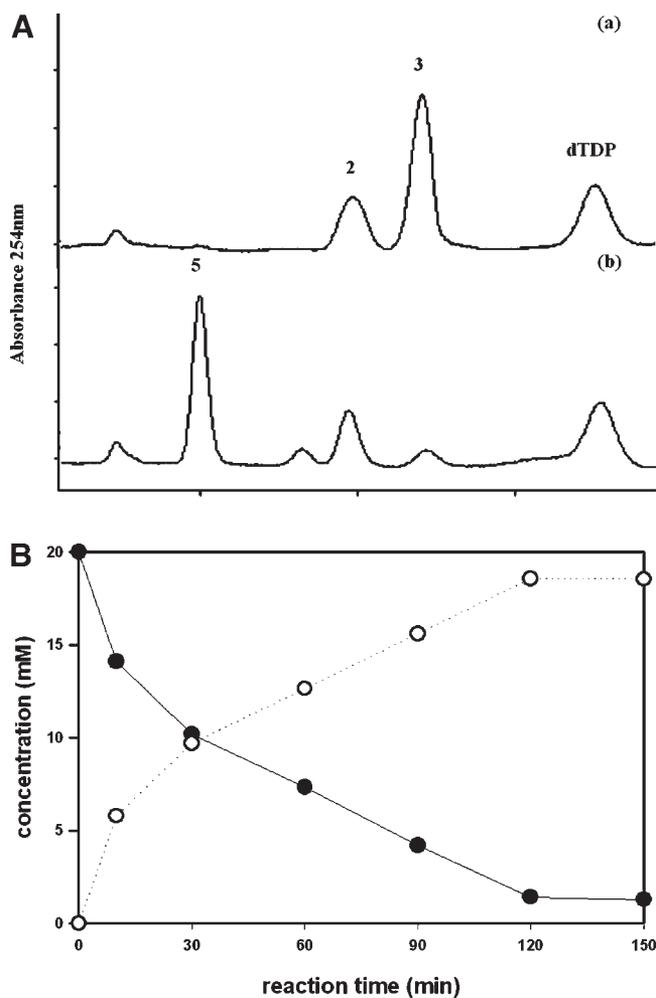


Figure 3. Determination of the net activity of putative dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase. **A:** HPLC profile of the biosynthesis of dTDP-L-rhamnose from dTKDG. **a:** chromatogram at 0 min, **(b)** chromatogram at 120 min. **B:** Reaction profile. ● dTKDG, ○ dTDP-L-rhamnose.

the initial condition. Peak3 exhibits the substrate dTKDG. Peak 2 and peak dTDP corresponded to dTDP-glucose and dTDP, respectively. We were always able to observe that dTKDG is gradually degraded into dTDP and dTDP-glucose due to its instability.

As the reaction goes on, the peak of dTKDG (peak 3) was diminished and the peak of dTDP-L-rhamnose (peak 5) rose up (Fig. 3A(a)). Since there was no commercially available standard of dTDP-L-rhamnose, analysis of the peak 5 was performed by ESI-MS. The fraction showed a peak at m/z 547 (data not shown), which coincides with the theoretical mass of dTDP-L-rhamnose. The identification of the product was further performed by NMR and confirmed, which are described in the next section. As shown in Figure 3B, the conversion yield of dTKDG to dTDP-L-rhamnose was about 92% after 120 min reaction. We measured the concentrations of dTKDG and dTDP-rhamnose by using the purified products as standards for HPLC analysis.

Above results demonstrate that dTDP-L-rhamnose was correctly synthesized from dTKDG by Mr7551 and Mr7553, proving that the putative genes really encode dTDP-4-keto-6-deoxy-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase, respectively.

One-Pot Synthesis of dTDP-L-Rhamnose from dTMP and Glucose-1-Phosphate

To demonstrate the production of dTDP-L-rhamnose from dTMP and glucose-1-phosphate as shown in the Figure 1, addition of the identified dTDP-4-keto-6-deoxy-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase to the four enzyme reaction system yielding dTKDG from dTMP and glucose-1-phosphate was performed. The reaction was conducted by incubating 20 mM dTMP, 80 mM glucose-1-phosphate, 60 mM acetyl phosphate, 10 mM NADH with the six enzyme extracts, that is, dTMP kinase, acetate kinase, dTDP-glucose synthase and dTDP-glucose 4,6-dehydratase, dTDP-4-keto-6-deoxy-glucose 3,5-epimerase, and dTDP-4-keto-rhamnose reductase, in 1 mL. Figure 4 shows the profile of the reaction. The overall yield of dTDP-L-rhamnose was about 82% based on initial dTMP consumed. Although the overall yield is lower than the conversion yield of dTDP-L-rhamnose from dTKDG by about 10%, the result indicates that dTDP-L-rhamnose can be directly produced from cheap substrates in one-pot reaction.

The one-pot reaction for preparative scale synthesis of dTDP-L-rhamnose was conducted in a volume of 20 mL, and the final product, dTDP-L-rhamnose, was purified from the reaction mixture using ion exchange column and desalting step. About 180 mg of white powder was finally obtained, corresponding to the overall yield of 60% based on initial

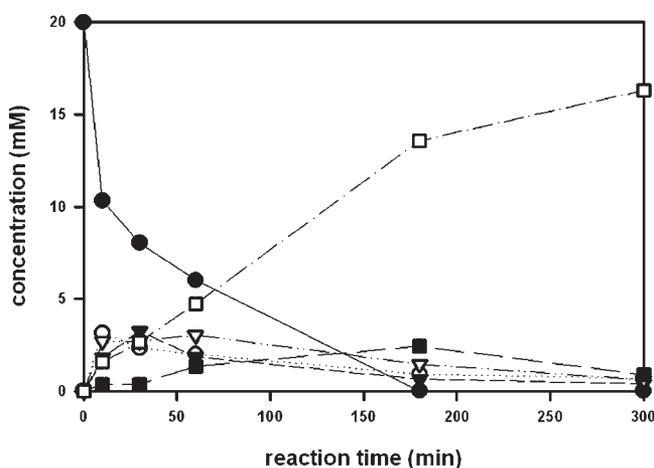


Figure 4. The reaction profiles of the biosynthesis of dTDP-L-rhamnose from dTMP using six enzymes. Reaction condition: 20 mM dTMP, 80 mM glucose-1-phosphate, 60 mM acetyl phosphate, 1 mM ATP, 20 mM MgCl₂, 10 mM NADH, approximately 2 units of TMP kinase, TDP-glucose synthase, dTKDG dehydratase, dTDP-4-keto-6-deoxy-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase (net activity), and 600 units of acetate kinase in 1 ml of Tris buffer (pH 7.5). ● dTMP, ○ dTDP, ▼ dTTP, □ dTDP-glucose, ■ TDP-4k-6d-glc, □ dTDP-L-rhamnose.

amount of dTMP. The product was analyzed by HPLC, giving approximately 95% purity. For the confirmation of dTDP-L-rhamnose, the purified product was also identified with 500 MHz ¹H-NMR and 500 MHz ¹³C-NMR. The proton–proton coupling constants (J) were determined from a DQF COSY spectrum (data not shown), (D₂O: δ 5.23 (d, 1H, H-1), δ 3.40(d, 1H, H-2), δ 3.66 (dd, 1H, H-3), δ 3.47 (dd, 1H, H-4), δ 4.10 (m, 1H, H-5), δ 1.32 (d, 3H, 5-Me), δ 6.35 (t, 1H, H'-1), δ 2.40 (dd, 2H, H'-2), δ 4.62 (dd, 1H, H'-3), δ 4.20 (d, 1H, H'-4), δ 4.20 (m, 1H, H'-5), δ 7.79 (s, 1H, H''-3), δ 1.94 (d, 3H, 5''-Me)) and 500 MHz ¹³C-NMR (D₂O: δ 95.97 (rhamnose C-1), δ 72.04 (rhamnose C-2), δ 73.17 (rhamnose C-3), δ 72.57 (rhamnose C-4), δ 70.77 (rhamnose C-5), δ 16.91 (rhamnose C-6), δ 85.61 (ribose C-1'), δ 38.74 (ribose C-2'), δ 71.28 (ribose C-3'), δ 86.35 (ribose C-4'), δ 65.88 (ribose C-5'), δ 152.13 (base C-2''), δ 166.91 (base C-4''), δ 111.13 (base C-5''), δ 137.75 (base C-6''), δ 12.12 (base CH₃)). These peaks agreed well with the previous findings (Marumo et al., 1992). Assignment of these resonances was verified by two-dimensional homonuclear H¹-NOESY (data not shown), H¹-COSY experiments (Fig. 5). The signal of H-4 (3.41 ppm) shows that the keto at C-4 happens to be reduced. The coupling constants also supported the orientations of 2-H, 3-H, 4-H, and 5-H being equatorial, axial, equatorial, and axial, respectively. The NMR results clearly showed the structure of dTDP-L-rhamnose.

DISCUSSION

In this study, we have shown that dTDP-L-rhamnose, the substrate of rhamnosyltransferase, can be synthesized on a preparative scale from dTMP and glucose-1-phosphate using six recombinant enzymes. To execute the reaction, we have functionally expressed and identified the putative genes in *M. luti* encoding dTDP-4-keto-6-deoxy-glucose 3,5-epimerase and dTDP-4-keto-rhamnose reductase. To our knowledge, this would be the first report showing the preparative enzymatic synthesis of dTDP-L-rhamnose from cheap substrate dTMP. We expect that our enzymatic preparative scale synthesis of dTDP-L-rhamnose may allow us to perform many related biological studies requiring large amount of dTDP-L-rhamnose such as rhamnosyltransferase catalyzed modification of antibiotics and synthesis of O-antigen. Moreover, the construction of in vitro metabolic pathway for dTDP-rhamnose may give us an opportunity to study easily on the inhibitory pathway for the synthesis of O-antigen found in many pathogens.

Our study also indicates that “genome based generation of in vitro metabolic pathway” can be indeed accomplished. Namely, we can design any interesting metabolic/enzymatic pathways, isolate the related enzymes from the genome of microorganism, and execute the designed reactions in vitro using recombinant enzymes. In practice, in vitro generation of biocatalytic pathway involving many enzymatic steps has been hampered by two main reasons. One is the shortage of information on the interesting enzyme such as gene sequence, and the other is uneasiness of the functional

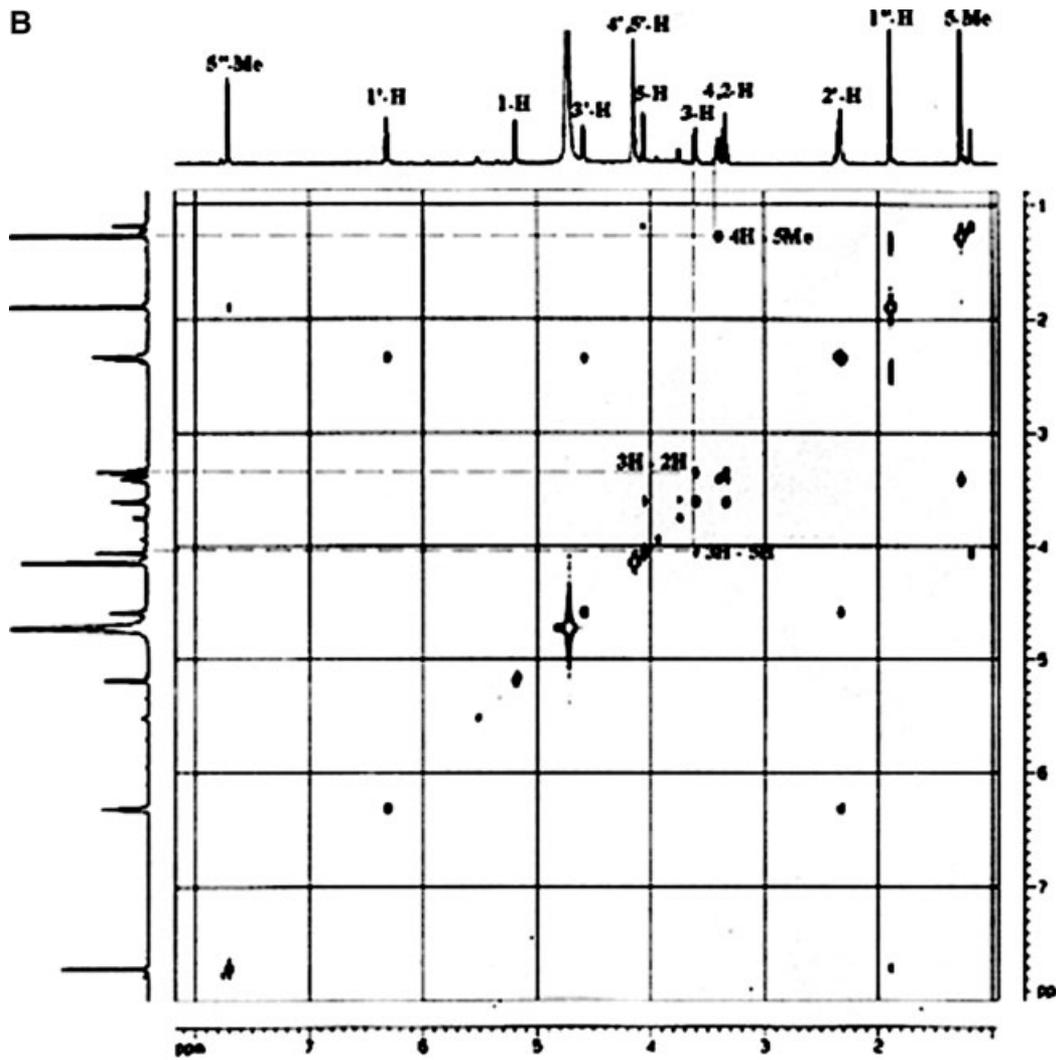
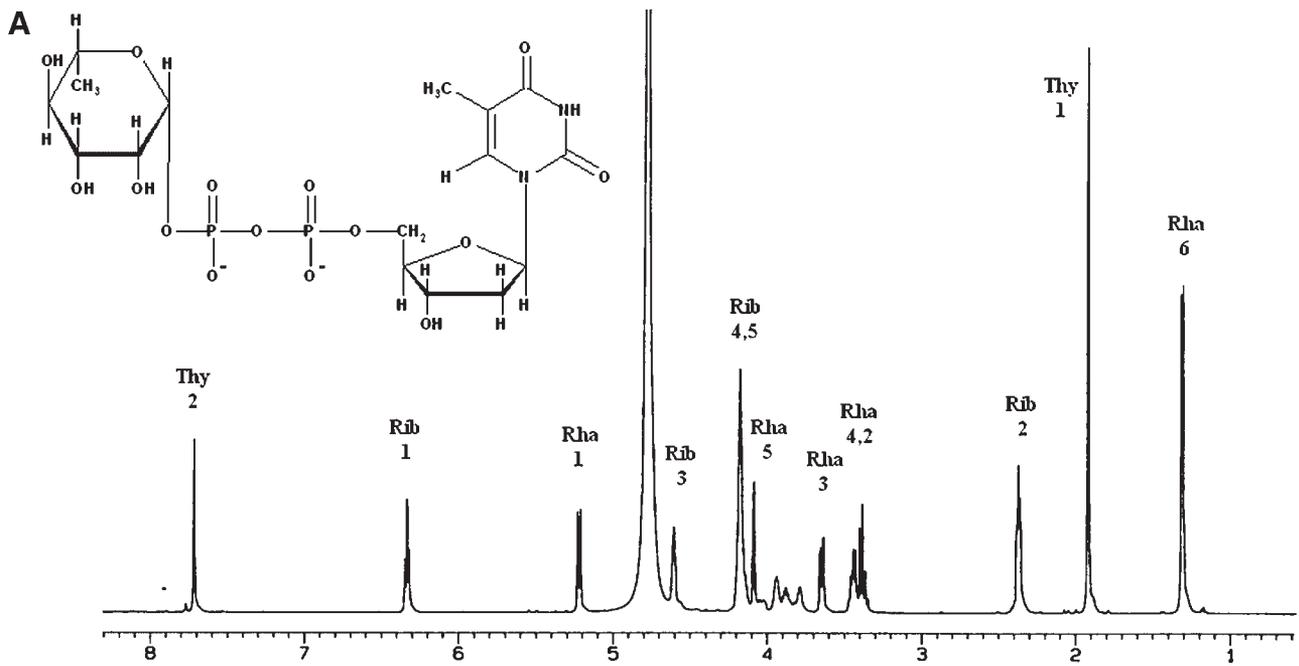


Figure 5. NMR analysis of the purified dTDP-L-rhamnose. A: ^{13}C -NMR, (B) ^1H -COSY analysis.

expression of the recombinant enzymes caused by the formation of inclusion bodies. However, rapid growth of biological database and development of new techniques in molecular biology are allowing us to overcome the drawbacks. For example, as shown in our study, the genome database allows us to access the required enzymes very easily and we can obtain functional proteins using appropriate gene expression system. In addition, molecular evolution technology is becoming an essential tool to enhance the availability of biocatalysts.

Even though we have suggested and demonstrated the enzymatic processes for dTMP-rhamnose, the process needs to be improved in order to become more practical and economical. For example, the result of one pot reaction shown in the Figure 4 was not performed under an optimal condition. The data show the considerable concentrations of the intermediates, such as dTDP, dTTP, dTDP-glucose, and dTKDG, indicating that the rates of the individual reactions are not well matched and the enzyme ratios are not proper. Although we have tried to optimize the reaction condition by varying the enzyme ratios, we have not achieved an improved result yet. We presume that there would be unknown effects caused by the use of enzymes as a form of cell extracts as well as inhibitions of sugar-nucleotide intermediates and product on enzymes.

This work was partially supported through a research program of Ministry of Commerce, Industry and Energy (MOCIE), and Nano Bioelectronics & Systems Research Center (ERC) of Seoul National University.

References

- Amann S, Dräger G, Rupprath C, Kirschning A, Elling L. 2001. (Chemo)enzymatic synthesis of dTDP-activated 2,6-dideoxysugars as building blocks of polyketide antibiotics. *Carbohydr Res* 335:23–32.
- Baneyx F, Mujacic M. 2004. Recombinant protein folding and misfolding in *Escherichia coli*. *Nat Biotechnol* 22:1399–1408.
- Bulter T, Elling L. 1999. Enzymatic synthesis of nucleotide sugars. *Glycoconjugate J* 16:147–159.
- Graninger M, Nidetzky B, Heinrichs DE, Whitfield C, Messner P. 1999. Characterization of dTDP-4-dehydrorhamnose 3,5-epimerase and dTDP-4-dehydrorhamnose reductase, required for dTDP-L-rhamnose biosynthesis in *Salmonella enterica Serovar Typhimurium* LT2. *J Biol Chem* 274:25069–25077.
- Haldimann A, Daniels LL, Wanner BL. 1998. Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. *J Bacteriol* 180:1277–1286.
- Kaneko T, Nakamura Y, Sato S, Asamizu E, Tabata S. 2000. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res* 7:331–338.
- Lee SG, Lee JO, Yi JK, Kim BG. 2002. Production of cytidine 5-monophosphate N-acetylneuraminic acid using recombinant *E. coli* as a biocatalyst. *Biotechnol Bioeng* 80:516–524.
- Marumo K, Lindquist L, Verma N, Weintraub A, Reeves PR, Lindberg AA. 1992. Enzymatic synthesis and isolation of thymidine diphosphate-6-deoxy-D-xylo-4-hexulose and thymidine diphosphate-L-rhamnose. *Eur J Biochem* 204:539–545.
- Oh JM, Lee SG, Kim BG, Sohng JK, Liou KK, Lee HC. 2003. Production of dTDP-4-keto-6-deoxy-D-glucose from dTMP and glucose-1-phosphate. *Biotechnol Bioeng* 84:452–458.
- Steffensky M, Muhlenweg A, Wang ZX, Li SM, Heide L. 2000. Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCBI 11891. *Antimicrob Agent Ch* 44:1214–1222.
- Varki A. 1993. Biological roles of oligosaccharides: All of the theories are correct. *Glycobiology* 3:97–130.