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One-pot enzymatic synthesis of deoxy-thymidine-diphosphate (TDP)-2-deoxy-α-D-glucose using phosphomannomutase

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ABSTRACT

Production of deoxy-thymidine-diphosphate (TDP)-sugars as substrates of glycosyltransferases, has been one of main hurdles for combinatorial antibiotic biosynthesis, which combines sugar moiety with aglycon of various antibiotics. Here, we report the one-pot enzymatic synthesis of TDP-2-deoxy-glucose employing high efficient TMP kinase (TMK; E.C. 2.7.2.12), acetate kinase (ACK; E.C. 2.7.1.21), and TDP-glucose synthase (TGS; E.C. 2.7.7.24) with phosphomannomutase (PMM; E.C. 5.4.2.8). In this study, replacing phosphoglucomutase (PGM; E.C. 5.4.2) by PMM from *Escherichia coli* gave four times higher specific activity on 2-deoxy-6-phosphate glucose, suggesting that the activity on 2-deoxy-glucose-6-phosphate was mainly affected by PMM activity, not PGM activity. Using an *in vitro* system starting from TMP and 2-deoxy-glucose-6-phosphate glucose, TDP-2-deoxy-glucose (63% yield) was successfully synthesized. Considering low productivity of NDP-sugars from cheap starting materials, this paper showed how production of NDP-sugars could be enhanced by controlling mutase activity.

1. Introduction

In the status quo, various antibiotic compounds have been discovered and widely used [1]. As there are many effective antibiotics being found, there are also increasing numbers of antibiotic resistant-bacteria, and the daunting speed of resistance to new antibiotics seem to exceed the speed of development of new antibiotics [2,3]. *In vivo* and *in vitro* combinatorial biosynthesis, which combines sugar moiety with a glycon of various antibiotics and changed the diversity of glycosylated antibiotics, has been proposed to speed up the development of new antibiotics [4,5]. However, it still has several drawbacks on efficacy, including poor activity of glycosyltransferase and limited availability of various activated glycan units [6,7].

Many macrolide antibiotics are composed of polyketide lactones substituted with 6-deoxy-hexose sugars [8], and antibiotic activities are typically determined by the number, nature, and relative distribution of the sugar moieties, which influence the pharmacokinetic properties of the drugs [9,10]. Most sugar units can be attached to aglycon when they are activated by nucleotide diphosphates, mainly with deoxy-thymidine-diphosphate (TDP) [11].

To make various TDP-glycans at a large-scale for combinatorial biosynthesis, our group and collaborators have been trying to produce various TDP-sugars enzymatically in an economic manner, and these attempts were quite successful with TDP-4-keto-6-deoxy-glucose, TDP-L-rhamonose and TDP-4-amino-4,6-dideoxy-D-glucose using acetate kinase and thymidine kinase systems in one pot [12–14].

In this report, TDP-2-deoxy-glucose, the precursor of TDP-4-keto-2,6-dideoxy-glucose to oliose oleandrose and oliose, was produced by enzymatic one-pot synthesis from TMP and 2-deoxy-6-phosphate-glucose, employing acetate kinase (ACK) and TMP kinase (TMK), phosphomannomutase (PMM), and TDP-glucose synthase (TGS) (Fig. 1). Introduction of PMM from *Escherichia coli* K12 showed four times higher specific activity than previously reported PGM on 2-deoxy-6-phosphate-glucose with same amount of substrates [15]. This could increase the conversion from 2-deoxy-6-phosphate-glucose to 2-deoxy-1-phosphate glucose and make TDP-2-deoxy-glucose more efficiently.

Abbreviations: TDP, deoxy-thymidine-diphosphate; TMP, deoxy-thymidine-monophosphate; TMK, deoxy-thymidine-monophosphate kinase; ACK, acetate kinase; TGS, deoxy-thymidine-diphosphate glucose synthase/glucose-1-phosphate thymidylytransferase; *E. coli*, *Escherichia coli*; PMM, phosphomannomutase; PGM, phosphoglucomutase; TTP, deoxy-thymidine-triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; Pi, phosphate; Acetyl Pi, acetyl phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate.

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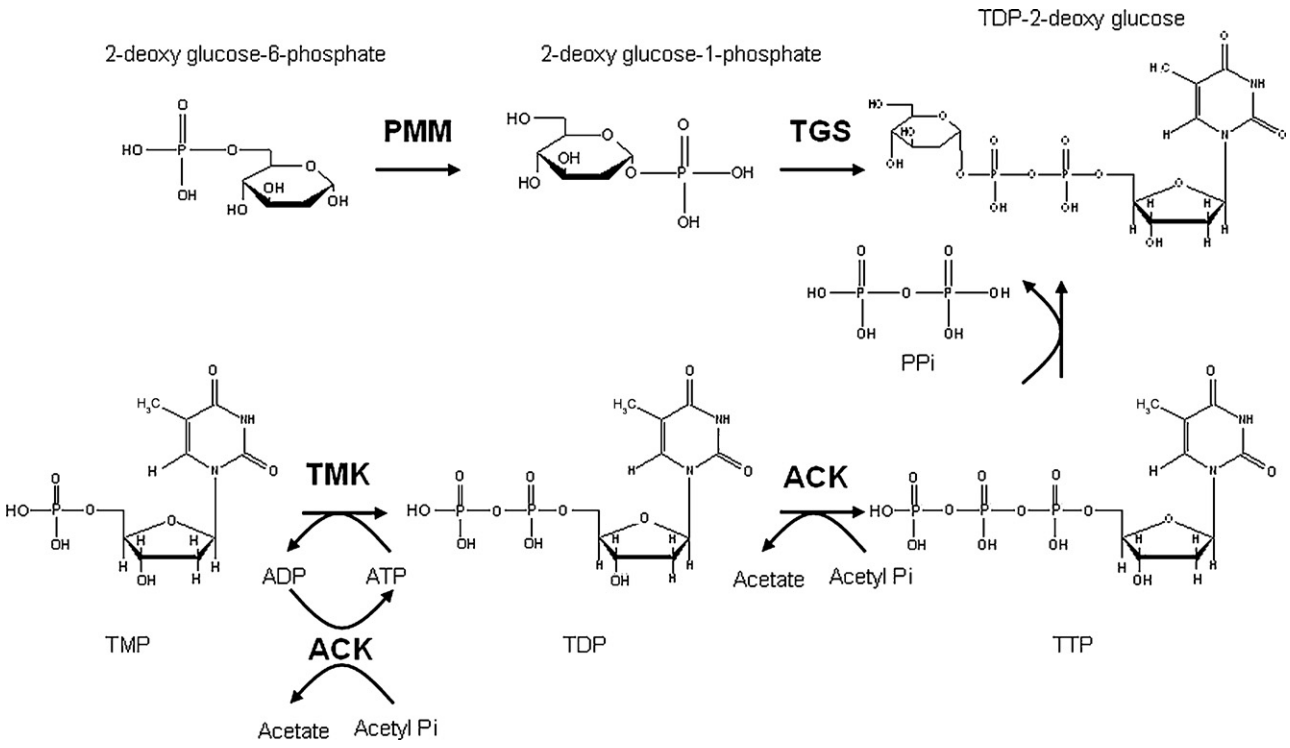

Fig. 1. A diagram of the biosynthesis of TDP-2-deoxy-glucose from 2-deoxy-glucose-6-phosphate and TMP with TMK, ACK, PMM and TGS.

2. Experimental methods

2.1. Bacterial strains, plasmids and chemical reagents

Chemical reagents used in this study were purchased from Sigma (MO, USA). *E. coli* BL21 (DE3) was from Novagen (WI, USA), and pET24ma was kindly donated by Dr. Hiroshi Sakamoto (Pasteur Institute, Paris, France). TDP-D-glucose was supplied by GeneChem (Daejeon, Korea) [12]. PMM was newly amplified using the chromosomal DNA of *E. coli* K12 as a template and cloned into pET24ma to give pYH0501. Other bacterial strains and plasmids used in this work are listed in Table 1.

2.2. Expression and preparation of enzymes

pYH0501 was transformed into *E. coli* BL21 (DE3). The cells were aerobically grown in 50 ml of Luria Bertani (LB) medium. Cells harboring each plasmid gene were grown to an OD_{600} of 0.6 at 30 °C, and then 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) was used for the induction. After 12 h, bacterial culture was harvested and pelleted. Crude extracts were prepared by the sonication of the cell suspension. For the enzyme assay, cell extracts were purified by His-tag agarose beads (Invitrogen, US). TMK, ACK, TGS were prepared as previously reported [12]. For the multiple enzyme reaction, each gene was expressed under optimal conditions and all the expressed protein was analyzed by 12% SDS-PAGE (data not shown).

Table 1
Plasmids used in this study.

Plasmid	Relevant information	Source or reference
pET24ma	p15A replication origin, T7 lac promoter, C-terminal his-tag coding	[22]
pYH0504	pET15b carrying PCR product of TMP kinase (TMK) from *E. coli* K12	[22]
pYH0503	pET24ma carrying PCR product of acetate kinase (ACK) from *E. coli* K12	[22]
pYH0502	pET24ma carrying PCR product of TDP-glucose synthase from *E. coli* K12	[22]
pYH0505	pET24ma carrying PCR product of TDP-glucose synthase from *Salmonella typhimurium*	This work
pYH0501	pET24ma carrying PCR product of Phosphomannomutase (PMM) from *E. coli* K12	This work

2.3. Enzyme activity assays

The PMM and TGS activities were identified by determining the net activity from 2-deoxy-glucose-6-phosphate and TTP. As a control, TGS from *Salmonella typhimurium* and phosphoglucomutase (PGM) from rabbit muscle (Sigma, USA) were used [15]. A reaction mixture (pH 7.5, volume: 100 μl) containing 10 mM TTP, 60 mM 2-deoxy-6-phosphate-glucose, 3 μM glucose-1,6-diphosphate, 5 mM $MgCl_2$, 20 mM Tris–HCl buffer, and 0.17 unit of TGS cell extracts was incubated at 37 °C. To compare PGM and PMM activities, 20 μg of PGM and PMM were used with same amount of TGS. One unit for PGM and PMM was defined as the amount of enzyme that produces 1 μ mol mannose-6-phosphate in 5 min at pH 7.4 and room temperature (15 °C), and one unit for TGS was defined as the amount catalyzing the formation of TDP-2-glucose. Twenty μg of PGM and PMM amount to 0.005 and 0.1 units of enzyme, respectively. The reaction was monitored by HPLC as described below. Likewise, to examine pH, buffer effect, metal effect and TMP/2-deoxy-6-phosphate-glucose ratio, the same concentration and reaction conditions were used with different pH, type of buffer, metal ion concentration, 2-deoxy-6-phosphate-glucose concentration, respectively.

2.4. Kinetic parameters of PMM from E. coli

All experiments were performed as previously published method [16]. Briefly, for the PMM assay, 1 μl of soluble protein (4.47×10^{-7} M) of purified soluble protein was added to a reaction buffer consisting of 50 mM Tris–HCl (pH 7.8), 5 μl of glucose-

6-phosphate-dehydrogenase (Roche, Germany), 1 mM β-NADP, 0.1 mM D-glucose-1,6-diphosphate, 5 μl of phosphomanno isomerase (Sigma, USA), 10 μl of phosphogluco isomerase (Roche, Germany), and 100 mM of $MgCl_2$ in a total volume of 100 μl. For the PGM assay, phosphomanno isomerase and phosphogluco isomerase were left out of the reaction mixture with same volume of PMM assay. Each assay was measured over a total of 5 min at 25 °C with 96-well plate and multiscanner (Thermo Electron Corp., Finland).

2.5. Biosynthesis and purification of TDP-2-deoxy-glucose from TMP and 2-deoxy-glucose-6-phosphate

Optimization reactions for TDP-2-deoxy-glucose were performed at pH 7.5 using 100 μl of 50 mM Tris–HCl buffer containing TMP, 2-deoxy-glucose-6-phosphate, acetyl phosphate, ATP, $MgCl_2$, and cell extracts for four enzymes at 37 °C. Ten μl of the sample was withdrawn, diluted with the same buffer (1:10), and the reactions analyzed by HPLC measurement.

For the preparative synthesis of TDP-2-deoxy-glucose, the reaction was carried out in 50 ml of reaction volume. The initial concentrations of TMP, ATP, acetyl phosphate, 2-deoxy-glucose-6-phosphate, glucose-1,6-diphosphate, and $MgCl_2$ were 10 mM, 2 mM, 30 mM, 60 mM, 3 μM, and 2 mM, respectively. After reacting for 3 h, the reaction mixture was boiled for 3 min, and the proteins were removed from the reaction mixture by ultrafiltration. Then, the remaining protein was removed by filter (0.45 μm, Gelman, USA). The product was purified using anion exchange (Dowex 1 × 2, Cl^-) and gel filtration (Sephadex G-15) columns (Supplementary Fig. 1). The lyophilized product was characterized with ^1H NMR, and its purity was analyzed by HPLC [13].

2.6. Instrumental analysis

Reaction substrates and products were analyzed by HPLC using an anion exchange analytical column (Carbopac PA1, 4.6 mm × 250 mm, 5 μm particle size, Dionex Associates, Netherlands) using isocratic 200 mM ammonium dipotassium phosphate buffer (pH 7.5) with a flow rate of 1 ml/min. The eluted nucleotides were monitored by absorbance at 254 nm. Retention times at this condition were as follows: TMP (3.1 min), TDP (12.5 min), TTP (90.0 min), TDP-2-deoxy-glucose (6.5 min).

To identify the final product, the purified products were dissolved in 700 μl D_2O (99.9 atom % D) and analyzed by NMR. ^1H were recorded with a Varian 300-MHz NMR spectrometer at a probe temperature of 298 K. Chemical shifts were expressed in ppm relative to an internal HOD signal (4.80).

Mass analysis (ESI-MS) for product was performed with LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corp, USA) with standard Thermo ESI source in negative ion mode at the spray voltage of 5 kV. Sample was infused by syringe pump at a rate of 5 μl min^{-1} and nebulized with dry nitrogen gas. The heated capillary was maintained at a temperature of 270 °C. The maximum ion collection time was set to 50 ms and five microscans were averaged per scan [17].

3. Results and discussions

3.1. Preparation of enzymes in E. coli

The molar ratio of glucose-1-phosphate to TMP and activity of TGS were the most important factors in the production of TDP-4-keto-6-deoxy-glucose [12] because of the low equilibrium constant of TGS and the instability of glucose-1-phosphate. To improve

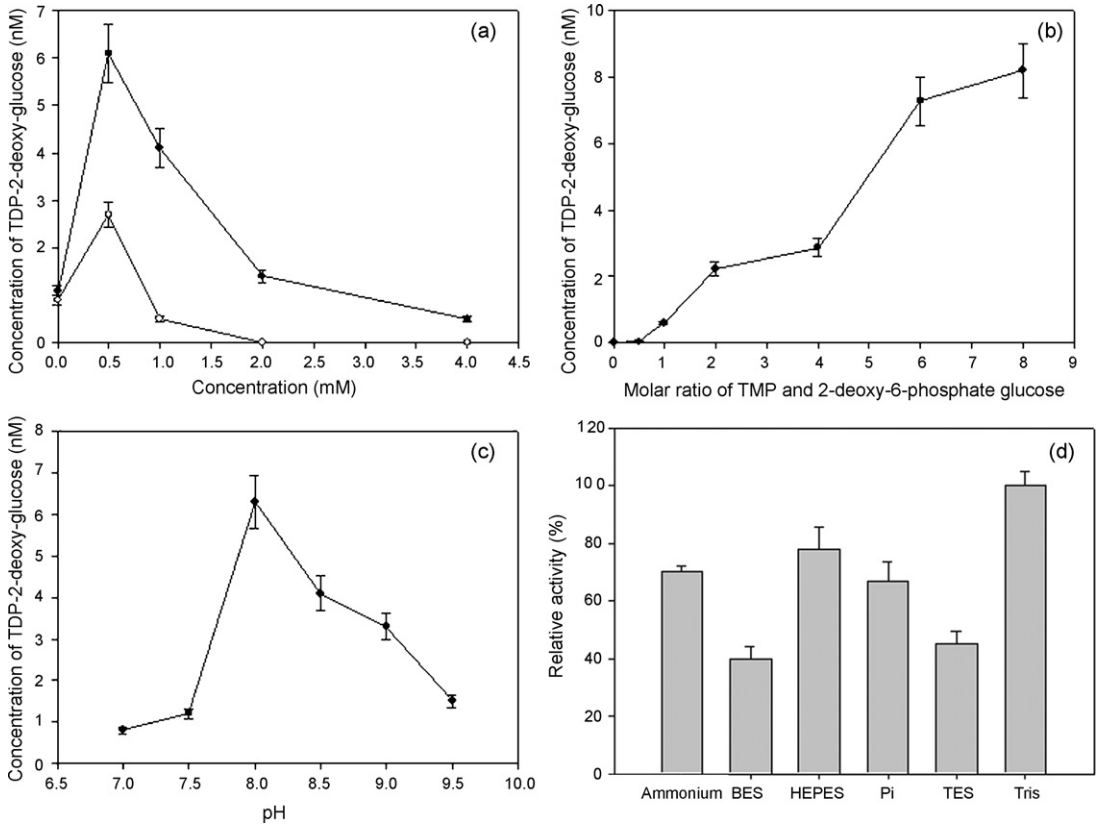

Fig. 2. Optimization of one-pot system. (A) The effects of metal ion concentration, Mg^{2+} (●) and Co^{2+} (○) on the production TDP-2-deoxy-glucose. (B) The molar ratio of TMP and 2-deoxy-6-phosphate-glucose. (C) The effect of pH and D. different types of buffers. Relative activities are based on Tris–HCl buffer, ammonium (ammonium bicarbonate buffer), BES, HEPES, pi (potassium phosphate buffer), and Tris (Tris–HCl buffer).

the production of TDP-2-deoxy-glucose, most of our attentions were focused on increasing the rate of producing 2-deoxy-glucose-1-phosphate from 2-deoxy-glucose-6-phosphate with PGM to increase the molar ratio of 2-deoxy-glucose-1-phosphate to TTP.

So far, there was only one report on the use of PGM from rabbit muscle for the production of 2-deoxy-glucose-1-phosphate [15] and its kinetic parameters with $K_{cat}/K_m = 2.550\,min^{-1}\,\mu M^{-1}$ for glucose-1-phosphate and, $K_{cat}/K_m = 5\,min^{-1}\,\mu M^{-1}$ for mannose-1-phosphate [18]. This enzyme has high catalytic activity on glucose-1-phosphate, but less activity on mannose-1-phosphate. Considering the structural similarities of 2-deoxy-glucose-6-phosphate to mannose-6-phosphate like glucose-6-phosphate, phosphomannomutase (PMM), which prefers mannose to PGM, also could to be another candidate enzyme for phosphate transfer by mutase. However, there has been no direct report on the kinetic value of the enzyme reaction of 2-deoxy-glucose-6-phosphate or 2-deoxy-glucose-1-phosphtae by PGM or PMM. For this reason, PMM from *E. coli* K12 was searched and examined among easily accessible strains.

Kinetic values of PMM were calculated as $K_{cat}/K_m = 130\,min^{-1}\,\mu M^{-1}$ for mannose-1-phosphate, but no activity for glucose-1-phophate. By the introduction of PMM to the production of TDP-2-deoxy-glucose, it improved the specific activity on 2-deoxy-glucose-6-phosphate than PGM by almost four times in a 2 h reaction by monitoring the amount of TDP-2-deoxy-glucose with HPLC (data not shown), suggesting the activity on 2-deoxy-6-phosphate-glucose was mainly affected by phosphomannose activity. It might be possible to add more PGM for enzymatic synthesis in one pot to increase overall reaction, however, a small amount of PMM will be preferred.

In another approach to find the genes for TGS, TGS from *S. typhimurium*, which has been used in an earlier report [15], was cloned to compare the activity of TGS from *E. coli* K12. Under the conditions described in Experimental methods, the activities of PMM and PGM with TGSs from different strains with same amount of substrates were compared, respectively (data not shown).

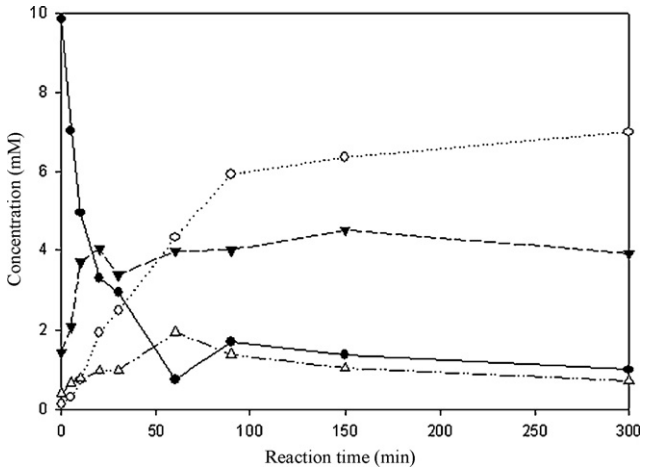

Fig. 3. The reaction profiles of the biosynthesis of TDP-2-deoxy glucose from TMP and 2-deoxy-6-phosphate-glucose using four enzymes and purification of products. TMP (●), TDP (▼), TTP (▽), TDP-2-deoxy-glucose (◯).

3.2. Optimization of one-pot reaction

The effects of representative metal ions for overall reaction were examined to improve productivity. The enzyme activity does not directly depend on the concentration of enzymes, but the ratio of the concentrations of the cofactor and the substrate [19].

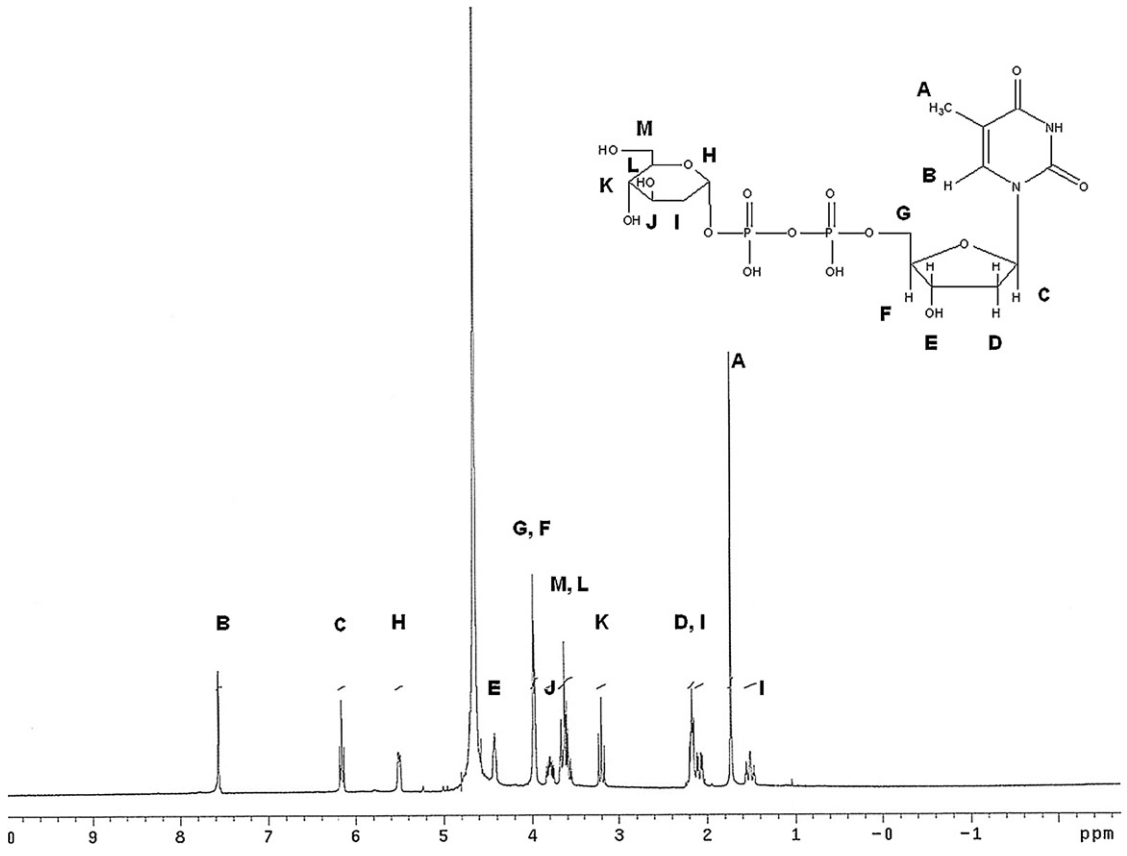

Fig. 4. ^{1}H NMR results of TDP-2-deoxy-glucose.

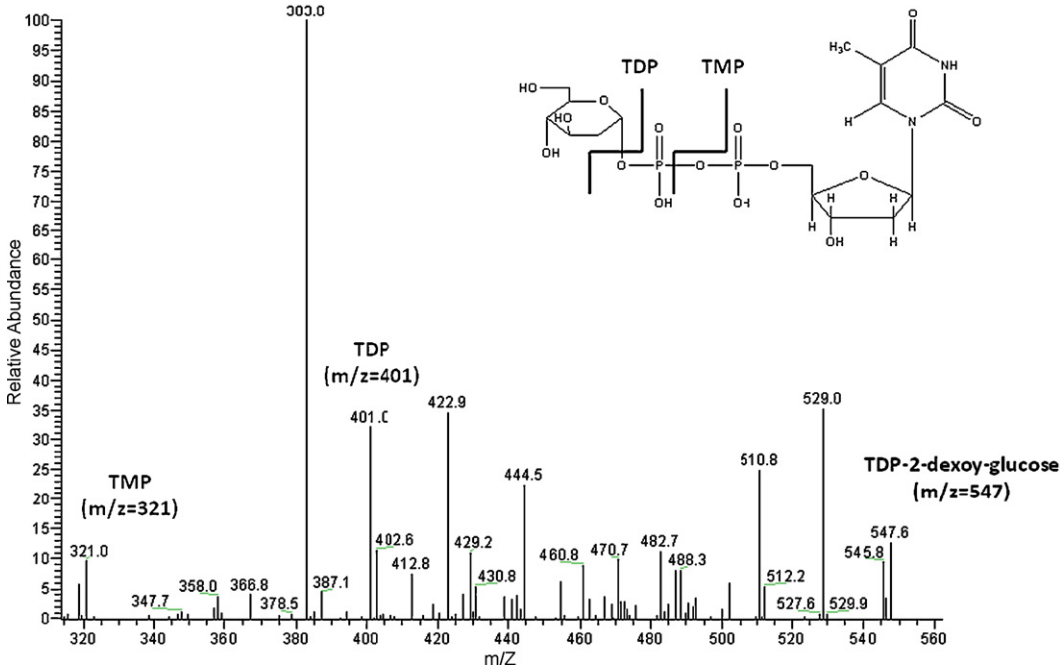

Fig. 5. MS/MS spectrum of TDP-2-deoxy-glucose (m/z = 547) in the negative mode.

Fig. 2A shows the optimal metal ion concentration for the overall reaction was Mg^{2+} 0.5 mM. As the main substrate molar ratio of TMP to 2-deoxy-6-phosphate-glucose increased up to eight, the amount of synthesized TDP-2-deoxy-glucose increased. Considering the greater cost of 2-deoxy-6-phosphate-glucose, the optimal conditions for large-scale production were needed, which had the maximum value of TDP-2-deoxy-glucose produced/2-deoxy-6-phosphate supplied (Fig. 2B). The maximum value was gained when the ratio of 2-deoxy-6-phosphate-glucose/TMP was six. Usually, the need of a high ratio of TMP/2-deoxy-6-phosphate-glucose was due to TMP being easily recycled whereas the mutase reaction is quite slow and rate limiting. In addition, TDP-glucose synthesis reaction is a reversible reaction and the equilibrium constant appears to be lower than the other two kinases and the instability of sugar-phosphate in the reaction buffer [12].

For the optimal pH and buffer, many buffers with concentration of 20 mM such as TES, BES, HEPES, ammonium bicarbonate, phosphate, and Tris–HCl, and various pH were examined (Fig. 2C and D). Among those, Tris–HCl buffer at a pH 8.0 produced the greatest activity.

3.3. One-pot synthesis

Based on all the preliminary experimental results, the one-pot enzymatic synthesis of TDP-2-deoxy-glucose was performed (Fig. 3). The product itself was very unstable and easily breakable to TDP and 2-deoxy-glucose-6-phosphate over time, and as a result, the reactions were stopped no later than 5 h. By using the one-pot system with highly active PMM, inhibitions by high concentration of TDP and TTP [20,21] could be avoided, and separation of TDP and TDP-2-deoxy-glucose was easier because of high productivity and low amounts of TDP, which was eluted at a similar time with TDP-2-deoxy-glucose (Fig. S1a). In addition, the high cost of TTP could be overcome with one-pot synthesis.

By one-pot reaction, 34 mg of products was finally obtained, which corresponds to a 63% yield based on the initial amount of TMP. The product was analyzed by HPLC, showing 97% purity, which has TDP with it as impurity. For the confirmation of TDP-2-deoxy-glucose, the purified product was also identified with 300 MHz ^{1}H NMR (Fig. 4) and MS/MS (Fig. 5). ^{1}H NMR (D_2O, 300 MHz) 7.57 (s, 1H (B)), 6.16 (app t, 1H, J = 5.7 Hz (C)), 5.52 (app d, 1H, J = 4.2 Hz (H)), 4.42 (m, 1H (E)), 3.98 (m, 1H (F)), 3.97 (m, 2H (G)), 3.80 (ddd, 1H, J = 9.1, 8.9 and 4.7 Hz (J)), 3.67 (m, 2H (M)), 3.66 (m, 1H (L)), 3.20 (app t, 1H, J = 8.9 Hz (K)), 2.17 (m, 2H (D)), 2.12 (app d, 1H, J = 11.8 and 4.7 Hz (I)), 1.73 (s, 3H (A)), 1.51 (ddd, 1H, J = 11.8, 9.1 and 4.2 Hz (I)).

In conclusion, enzymatic synthesis of TMP and 2-deoxy-6-phosphate-glucose could successfully produce large amount of TDP-2-deoxy-glucose in one-pot by employing phosphomannomutase, which showed four times higher activity than the previously reported method [15]. To further improve this system, hexose kinase, which can transfer the phosphate group to 2-deoxy-glucose is currently being studied. This enzyme will make it possible to use a more inexpensive starting material, 2-deoxy-glucose. Again, by introduction of PMM, the overall production will be enhanced and cheaper process will be achieved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2009.11.008.

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