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View Article Online
DOI: 10.1039/C6CC08366H

Journal Name

COMMUNICATION

One-Pot Four-Enzyme Synthesis of Thymidinediphosphate-L-rhamnose

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Abstract. A new, robust one-pot four-enzyme synthetic method was developed for thymidinediphosphate-L-rhamnose starting from D-glucose-1-phosphate. The enzymes, Glc-1-P thymidylyltransferase, dTDP-Glc-4,6-dehydratase, dTDP-4-keto-6-deoxy-Glc-3,5-epimerase and dTDP-4-keto-Rha reductase were derived from *Streptococcus pneumonia* serotype 23F, expressed in *Escherichia coli,* and studied in detail to provide the first direct evidence for their functions.

L-Rhamnose (Rha) is a common component of many bacterial polysaccharides, e.g., lipopolysaccharides[1,2] and extracellular,[3] capsular[4] and cell wall polysaccharides,[5,6] which play a pivotal role in various biological events. To study these polysaccharides and related glycoconjugates, it is necessary to have access to the molecules in structurally homogeneous and defined forms, which is difficult to achieve via isolation of natural products. Thus, chemical and enzymatic synthesis of polysaccharides and their analogues has been a hot topic. In this regard, enzymatic synthesis is especially attractive due to the complex structure of the synthetic targets and the difficulty to create some glycosidic bonds, such as that of β-linked Rha, via chemical glycosylation. For enzymatic synthesis of Rha-containing glycoconjugates, thymidine diphosphate-L-rhamnose (dTDP-Rha) is a key substrate,[7-13] used by rhamnosyltransferases (Rha-Ts) as the rhamnosyl donor to be attached to sugar acceptors.[14]
However, dTDP-Rha is not easily accessible, and its chemical synthesis remains a significant challenge.[13] As a result, much recent research effort has been put on its enzymatic synthesis. dTDP-Rha biosynthesis is conserved in bacteria,[15] involving enzymes such as RmlA, RmlB, RmlC, and RmlD.[16-26] Marumo,[13] Graninger[27] and Steiner[28] labs reported an enzymatic synthesis of dTDP-Rha utilizing dTDP-D-glucose (dTDP-Glc), a biosynthetic intermediate of dTDP-Rha, and crude enzymes.[29-32] However, this method is not suitable for large scale synthesis since dTDP-Glc is expensive and difficult to obtain. A less costly synthesis for dTDP-Rha was developed by the use of six crude enzymes.[33] However, the quality of enzymes was uncontrollable, and the product purification was challenging. James and coworkers[34,35] described a two-step four-enzyme synthesis, but the reactions were poorly characterized. For example, the reaction yield and the product purity were not described in the paper. Moreover, this synthesis needed two purification steps, which is still demanding. Clearly, a more robust synthetic method for dTDP-Rha using well-characterized enzymes and low-cost and easily accessible starting materials is highly desirable.
We report herewith a new, simple, and effective one-pot four-enzyme synthesis of dTDP-Rha from commercial D-glucose-1-phosphate (Glc-1-P) and thymidine triphosphate (dTTP). The enzymes used included Glc-1-P thymidylyltransferase Cps23FL, dTDP-Glc-4,6-dehydratase Cps23FN, dTDP-4-keto-6-deoxy-Glc-3,5-epimerase Cps23FM, and dTDP-4-keto-Rha reductase Cps23FO derived from *Streptococcus pneumonia* serotype 23F. Although the functions of Cps23FL, Cps23FN, Cps23FM and Cps23FO were proposed,[36] no direct biochemical evidence for their activities or detailed studies and synthetic applications of the pure enzymes have been reported previously.

Expression and purification of the enzymes involved in dTDP-Rha biosynthesis in *S. pneumonia* serotype 23F. Capsular polysaccharides derived from various *S. pneumonia* serotypes contain Rha,[37] and their biosynthesis uses dTDP-Rha as the Rha donor.[24,38] In turn, the biosynthesis of dTDP-Rha starting from Glc-1-P (Scheme 1)[7,13,28] involves four enzymes, including Glc-1-P thymidylyltransferase, dTDP-Glc-4,6-dehydratase, dTDP-4-keto-6-deoxy-Glc-3,5-epimerase, and dTDP-4-keto-Rha reductase.
In *S. pneumonia* type 23F, proteins Cps23FL, Cps23FN, Cps23FM, and Cps23FO were proposed to correspond to above enzymes, respectively.[36] To study these gene products in detail and their applications to dTDP-Rha synthesis *in vitro*, we cloned *cps23FL, cps23FM, cps23FN,* and *cps23FO* genes from *S. pneumonia* 23F by the conventional protocols, using primers listed in Table S1,

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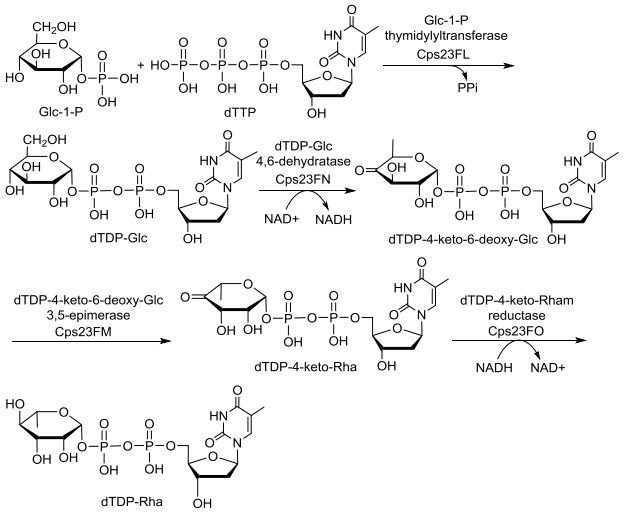
Electronic Supplementary Information (ESI) available: Experimental procedures, primers used for gene cloning, protein analysis results, enzyme reaction condition optimization and kinetics results, product MS and NMR spectra. See DOI: 10.1039/x0xx00000x

and used them to express corresponding proteins in *Escherichia coli*. In addition, a His-6 tag was introduced to the N-terminus of these proteins to facilitate their purification. SDS-PAGE and western blot studies (SI Figures S1A and S1B) of the purified proteins showed that their molecular weights (34.3, 41.1, 24.4 and 34.5 KD, respectively) agreed with the predicted molecular weights of Cps23FL, Cps23FN, Cps23FM and Cps23FO. Figure S1 also revealed that the proteins were essentially homogeneous. The purified proteins were also analysed with the BCA protein quantification kit to determine the protein expression levels of Cps23FL, Cps23FN, Cps23FM, and Cps23FO, which were 107, 91, 65, and 94 mg/litter, respectively. The pure proteins were stable in Tris-HCl buffer containing 30% glycerol at pH 7.5 and showed no activity loss after storing at -20 ^{o}C for 2 months.



Scheme 1. Biosynthetic pathway of dTDP-Rha in *S. pneumonia*

Biochemical studies of Cps23FL, Cps23FM, Cps23FN, and Cps23FO. Initially, we briefly evaluated the activities of the obtained proteins by indirect assays. It was found that after adding Cps23FL and Cps23FN, the mixture of Glc-1-P, dTTP, and NAD^+ showed a significantly increased absorbance at the wavelength of 320 nm than the control groups containing no or only one enzyme (Figure 1A), indicating the formation of 4-keto sugar from the first two reactions depicted in Scheme 1. Furthermore, upon treatment with Cps23FM and Cps23FO, the mixture derived from the above reactions showed a decreased absorbance at 340 nm than the controls (Figure 1B), indicating the consumption of NADH by the last reaction in Scheme 1. These results suggested that Cps23FL, Cps23FN, Cps23FM, and Cps23FO might possess the desired enzymatic activities**.**

Next, each enzyme was examined more closely. To confirm the activity of Cps23FL, the product of its reaction with Glc-1-P and dTTP was isolated by Q anion exchange chromatography. The ion peak at *m/z* 563.0705 of its high resolution mass spectrum (HRMS, Figure S2) agreed with the molecular weight of dTDP-Glc (563.0679 for $C_{16}H_{25}N_2O_{16}P_2$ $[M-H]^{-1}$), and its ^{1}H and ^{13}C NMR spectra (Figure S3) were the same as that in the literature.[39,40] Therefore, both MS and NMR data confirmed the product as dTDP-Glc. The reaction yield was about 86% based on Glc-1-P. This study proved unambiguously the activity of Cps23FL as an enzyme to catalyze the conversion of Glc-1-P to dTDP-Glc.

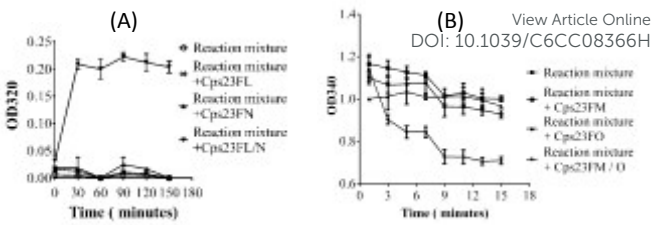


Figure 1. Results of indirect assays of the enzymatic activities of Cps23FL, Cps23FN, Cps23FM, and Cps23FO. (A) Reaction between Glc-1-P and dTTP in the presence of Cps23FL or Cps23FN alone or Cps23FL plus Cps23FN. (B) Conversion of product obtained with Cps23FL and Cps23FN in the presence of Cps23FM or Cps23FO alone or both Cps23FM and Cps23FO. Each data point represents the mean ± standard error of three experiments

To optimize the reaction conditions for Cps23FL, it was treated with Glc-1-P and dTTP at varied pH, temperature, and metal ion concentration, with heat-deactivated Cps23FL as a control. The reaction was monitored by the malachite green colorimetric assay[41] detecting the generated pyrophosphate (PPi) upon its degradation by an inorganic pyrophosphatase YIPP to generate phosphate. To avoid potential influences of YIPP concentration on the assays, YIPP was utilized in large excess. As depicted in Figures S4A and S4B, Cps23FL had the maximal catalytic activity at pH 7.5 and 25 ^{o}C. However, Cps23FL tolerated a relatively wide range of temperature, as >80% of its activity retained from 16 to 40 ^{o}C. It was also found that divalent metal cations were required for its catalytic activity and the optimal cation was Mg^{2+} (Figure S4C). Moreover, the activity of Cps23FL reached its maximum at 0.63 to 40 mM of Mg^{2+} (Figure S4D). Thus, for future study and application of Cps23FL, its reaction conditions were set at pH 7.5, 37 ^{o}C, and 5.0 mM of Mg^{2+}.

To evaluate the reaction kinetics of Cps23FL, its K_m and V_{max} values for both substrates were determined. Initial velocities of the reaction under optimized conditions were obtained using varied dTTP concentrations (0.01～0.08 mM) and saturated Glc-1-P (1 mM) or varied Glc-1-P concentrations (0.05～0.40 mM) and saturated dTTP (1 mM). The data were employed to create Michaelis-Menten plots and derive K_m and V_{max}, which were found to be 9.57 ± 1.58 μM and 4.15 ± 0.16 μM/min for dTTP, and 18.04 ± 1.48 μM and 8.36 ± 0.01 μM/min for D-Glc-1-P, respectively. Comparing the K_m values of Cps23FL to that of RmlA derived from other bacteria, such as *Mycobacterium tuberculosis,*[42] *Salmonella enterica* LT2[43] and *Aneurinibacillus thermoaerophilus*[27] (0.02, 0.02, 0.03 mM for dTTP; 0.07, 0.11, 0.20 mM for D-Glc-1-P, respectively), the K_m value of Cps23FL for D-Glc-1-P was significantly lower, indicating that Cps23FL had higher affinity to D-Glc-1-P. However, there was not a drastic difference among the K_m values for dTTP.

To verify the enzymatic activity of Cps23FN, it was reacted with a mixture of Cps23FL, Glc-1-P, dTTP and NAD^+ under conditions described above. The product was obtained in a 71% yield after purification by HPLC and by a G10 column. Its HR MS (Figure S5) gave an ion peak at m/z 545.0546, which agreed well with the molecular weight of dT4k6dG ($C_{16}H_{22}N_2O_{15}P_2$ $[M-H]^-$, 545.0574). Its ^{1}H and ^{13}C NMR spectra (Figure S6) were also identical to that of the reported dT4k6dG.[44-46] Both HR MS and

NMR data proved the production of dT4k6dG and thus the dTDP-Glc-4,6-dehydratase activity of Cps23FN.

Subsequently, the catalytic property of Cps23FN was studied in detail by using pure dTDP-Glc as substrate. The reaction was examined by spectrophotometric analysis of resulting dT4k6dG, which had specific absorption at 320 nm under basic condition, caused by enediolate formed from the deprotonation of keto sugar.[27,33,47-49] In these experiments, mixtures of dTDP-Glc and NAD^+ were incubated with Cps23FN or the heat-deactivated enzyme (negative control), respectively, and then quenched with 1 M NaOH. We found that Cps23FN showed the highest catalytic activity at 37 °C and pH 7.5 (Figure S7), while various cations had a negligible impact on the reaction. Moreover, the initial velocities of Cps23FN-catalyzed reactions under optimal conditions were measured with different concentrations (0.1 ~1.0 mM) of dTDP-Glc. The results were used to obtain the K_m (0.86 ± 0.11 mM) and V_{max} (0.07 ± 0.004 mM/min) of Cps23FN. The higher K_m value of Cps23FN than that of RmlB from other bacteria, such as *S. enterica* LT2 (0.427 mM),[50] *M. tuberculosis* (0.050 mM)[48] and *E. coli* RmlB (0.070 mM),[51] indicated that Cps23FN had lower affinity to dTDP-D-glucose.

Detailed study of Cps23FM or Cps23FO individually turned out to be difficult since (1) Cps23FM alone without Cps23FO had a low rate of substrate conversion (*ca*. 3%) and (2) its product, TDP-4-keto-L-Rha, was not stable enough to be isolated.[52] Thus, the two enzymes were studied in conjugation. To confirm the enzymatic activities of Cps23FM and Cps23FO disclosed above, they were incubated with pure dT4k6dG and NAD^+/NADH. The reaction mixture was subjected to HPLC analysis, revealing a new product that was collected and characterized. Its HR MS showed a peak at m/z 547.0711 (Figure S8) that agreed with the calculated molecular weight of dTDP-Rha (547.0736 for $C_{16}H_{25}N_2O_{15}P_2$ $[M\text{-}H]^-$). Its ^1H, ^{13}C, and ^{31}P NMR spectra (Figure S9) were in accordance with the structure of dTDP-Rha.[13] The production of dTDP-Rha from this reaction proved ultimately the dTDP-4-keto-6-deoxy-Glc-3,5-epimerase and dTDP-4-keto-L-Rha reductase activities of Cps23FM and Cps23FO.

One-pot four-enzyme synthesis of dTDP-Rha from Glc-1-P. After the properties of Cps23FL, Cps23FN, Cps23FM, and Cps23FO were evaluated in detail and their enzymatic activities were confirmed, we explored their application to the synthesis of dTDP-Rha. As the optimized reaction conditions for these enzymes, such as pH (7.5) and temperature (37 °C), are similar, we were interested in a one-pot multiple enzyme synthetic protocol. This would have several advantages, e.g., reducing the number of purification steps and avoiding the isolation of relatively unstable intermediates.

Our initial synthetic design was to simultaneously mix all of the substrates, reagents, and enzymes in one pot, but it gave only a very low yield (~1%) of dTDP-Rha, probably because of the inhibition of dTDP-Rha formed from the reaction on Cps23FL.[53] We found that the reaction yields was significantly improved when the enzymes were added in two portions. First, a solution of Glc-1-P (3 mM), dTTP (5 mM), NAD^+ (0.1 mM), and NADH (6 mM) in a HEPES buffer (30 mM, pH 7.5) containing 5 mM of $MgCl_2$ was incubated with Cps23FL (2 μM, 68.6 mg/L) at 37 °C for 15 minutes. Excessive NADH was used here to drive the reaction equilibrium towards the final product later on. Thereafter, Cps23FL was removed by ultrafiltration, followed by adding Cps23FN, Cps23FM, and Cps23FO (2 μM, 82.2, 48.8 and 69.0 mg/L, respectively). The reaction was monitored with HPLC to reveal the generation of dTDP-Rha in a good yield (Figure 2). After completion, the reaction was quenched and the enzymes were removed by ultrafiltration. The final product was purified by HPLC, desalted on a G10 column, and then lyophilized to produce dTDP-Rha as a white solid. The overall isolated yield was 47% based on Glc-1-P. However, dTDP-Rha was not easily separated from side products such as dTMP and NADH by Q anion exchange chromatography. The reaction yield was further improved to 63% when the operation to remove Cps23FL by ultrafiltration was eliminated, probably as a result of decreased intermediate loss. It is also worth noting that using excessive dTTP (*ca*. 1.5 equiv) in the reaction was beneficial, because it not only improved the reaction yield but also simplified product purification by exhausting Glc-1-P that was difficult to separate from dTDP-Rha.

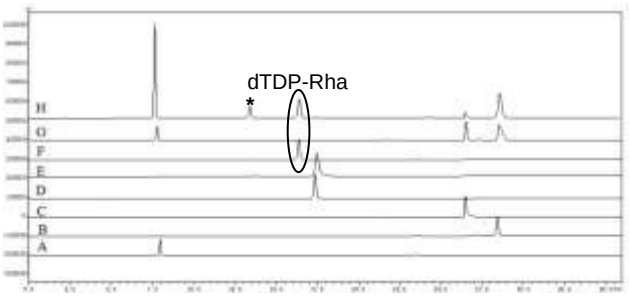


Figure 2. HPLC diagrams of (A) NAD^+, (B) NADH, (C) dTTP, (D) dTDP-Glc, (E) dT4k6dG, (F) dTDP-Rha, (G) reaction mixture of heat-deactivated enzymes, and (H) reaction mixture for one-pot four-enzyme synthesis of dTDP-Rha from Glc-1-P. *dTMP

This study has established a straightforward, efficient, and economical one-pot synthetic method for dTDP-Rha starting from inexpensive and commercially available Glc-1-P, by using enzymes cloned from *S. pneumonia* serotype 23F. Compared to the preparation methods reported in the literature, this strategy has several advantages. For example, in addition to the inexpensive starting material and abolition of intermediate isolations through one-pot synthesis, the reaction progress could be easily monitored and examined, and the final product purification was simple, due to the use of pure enzymes in the synthesis. Therefore, this synthetic method has the potential of being used for large scale synthesis. For instance, dTDP-Rha was prepared in hundred milligram scales in the authors' lab. Moreover, the enzymes involved, including Cps23FL, Cps23FN, Cps23FM and Cps23FO, were purified and examined in detail, which provided the first direct biochemical evidence for the functions of these gene products and formed the foundation for more in-depth studies. Overall, easy access to dTDP-Rha would facilitate Rha-T-based chemoenzymatic synthesis of Rha-containing oligosaccharides, polysaccharides, rhamnolipids,[54,55] and related conjugates which should be very useful for various biological studies and applications.

Acknowledgement.

This work was supported by grants from the National Natural Science Foundation of China (21672129 and 21472114).

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