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Enzymatic synthesis of dTDP-4-amino-4,6-dideoxy-D-glucose using GerB (dTDP-4-keto-6-deoxy-D-glucose aminotransferase)

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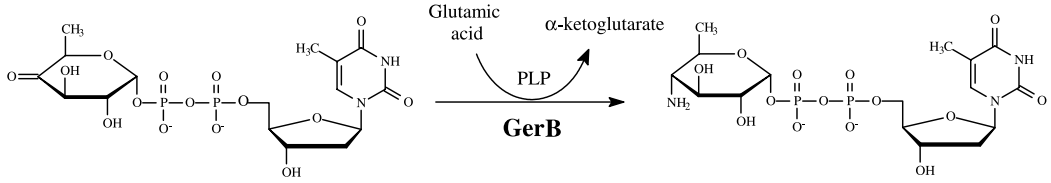
Abstract—Over-expressed GerB (dTDP-4-keto-6-deoxy-D-glucose aminotransferase) of *Streptomyces* sp. GERI-155 was used in the enzymatic synthesis of dTDP-4-amino-4,6-dideoxy-D-glucose (**2**) from dTDP-4-keto-6-deoxy-D-glucose (**1**).



dTDP-4-keto-6-deoxy-D-glucose (1) **dTDP-4-amino-4,6-dideoxy-D-glucose (2)**

Five enzymes including dTMP kinase (TMK), acetate kinase (ACK), dTDP-glucose synthase (TGS), dTDP-glucose 4,6-dehydratase (DH), and dTDP-4-keto-6-deoxy-D-glucose aminotransferase (GerB) were used to synthesize **2** on a large scale from glucose-1-phosphate and TMP. A conversion yield of up to 57% was obtained by HPLC peak integration given a reaction time of 270 min. After purification by two successive preparative HPLC systems, the final product was identified by HPLC and then analyzed by ^{1}H, ^{13}C, ^{1}H–^{1}H COSY NMR spectrometry.

Keywords: Aminotransferase; dTDP-4-Amino-4,6-dideoxy-D-glucose; dTDP-4-Keto-6-deoxy-D-glucose; L-Glutamic acid; *Streptomyces* sp. Geri-155

1. Introduction

Deoxyaminosugars comprise an important class of deoxysugar moieties synthesized by a variety of microorganisms including plants, fungi, and bacteria.[1] Bacteria, actinomycetes in particular, produce secondary metabolites with modified deoxysugar moieties, which have antibiotic and antitumor activities.[2,3] Deoxyaminosugars are usually formed via a transamination reaction, which results in the substitution of an amino group for a keto

Abbreviations: ACK, acetate kinase; DH, dTDP-glucose 4,6-dehydratase; HPLC, high performance liquid chromatography; IPTG, isopropyl-D-thiogalactopyranoside; PLP, pyridoxal phosphate; SAM, *S*-adenosylmethionine; TEAB, tetraethylammonium borate; TGS, dTDP-glucose synthase; TMK, dTMP kinase

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group. These deoxyaminosugars are crucial for the interactions between antibiotic molecules, which appear to play an important role in the function of the cellular membrane of the target organism.[4] Therefore, studies on the synthesis and attachment of deoxyaminosugars are important for the engineered biosynthesis of new antibiotics from bacteria. Bowers et al. reported the synthesis of dTDP-4-amino-4,6-dideoxy-D-glucose (**2**) from galactose using a 16-step procedure.[5] Alternative biosynthetic pathways for the biosynthesis of deoxyaminosugars have been proposed and several aminotransferases that catalyze the installation of an amino group on a deoxysugar have been studied. The aminotransferases studied include *N*-methyl-L-glucosamine (streptomycin),[6] D-desosamine (erythromycin),[7,8] D-desosamine (oleandomycin),[9] and D-mycaminose (tylosin).[10]

However, there are no reports on the enzymatic synthesis of **2**. We have recently isolated the mycinose gene cluster involved in the biosynthesis of dihydrochalocomycin from *Streptomyces* sp. KCTC 0041BP.[11] The GerB open reading frame was predicted to be an aminotransferase involved in the biosynthesis of **2**. The deduced amino acid sequence (426 aa) shares an identity of roughly 65% with a number of the aminotransferases in the GeneBank database. We report the cloning and expression of *gerB* in *Escherichia coli* BL21 (DE3) along with the enzymatic synthesis of **2** and its stereochemistry via ^{1}H, ^{13}C, and ^{1}H–^{1}H COSY NMR analysis.

2. Results and discussion

2.1. Identification of dTDP-4-keto-6-deoxy-D-glucose aminotransferase, GerB

The *gerB* gene is located in the region furthest upstream in the dihydrochalocomycin biosynthetic gene cluster. The deduced amino acid sequence of *gerB* showed a high degree of similarity to other putative genes within the GeneBank database; such as EryCIV (GeneBank accession no. AAB84075),[7] which is the aminotransferase involved in D-desosamine biosynthesis (62% identity); OleNI (GeneBank accession no. AAD55456),[9] the dehydratase involved in the transamination (66% identity); and DesI (GeneBank accession no. AAC68684),[12] the dehydrase involved in D-desosamine biosynthesis (61% identity). These results are shown in Figure 1. Liu and co-workers recently accomplished the biosynthesis of dTDP-D-desosamine using a two-step C-4 deoxygenation in which DesI was used to catalyze the C-4 transamination of dTDP-4-keto-6-deoxy-D-glucose (**1**) to generate **2** and in which DesII

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EryCIV   --MKRALTDLAIFGGPEAFLHTLYVGRPTVGDRERFFARLEWALNNNWLTNGGPLVREFE 58
OleNI    --MKRGVHDLALFGGDAAFLQPLYMGRPNTGDRKRLLDRLEWALDNRWLTNGGPLVREFE 58
GerB     MTLKRNVGDLAVFGGPSAFLQPLHVGRPNVGDRARFFDRVNWALDHQWLTNGGPLAREFE 60
DesI     --MKSALSDLAFFGGPAAFDQPLLVGRPNRIDRARLYERLDRALDSQWLSNGGPLVREFE 58

EryCIV   GRVADLAGVRHCVATCNATVALQLVLRASDVSGEVVMPSMTFAATAHAASWLGLEPVFCD 118
OleNI    QRIADLAGVRNCVATCNATAGLQLLLREAEVTGEVIMPSMTFVATAHAVRWLGLRPVFCD 118
GerB     QRVAELAGVRYCVSTCNATVALQLLARAADLSGEVIMPSLTFPATAQAFRWLGLTPVFCD 120
DesI     ERVAGLAGVRHAVATCNATAGLQLLAHAAGLTGEVIMPSMTFAATPHALRWIGLTPVFAD 118

EryCIV   VDPETGLLDPEHVASLVTPRTGAIIGVHLWGRPAPVEALEKIAAEHQVKLFFDAAHALGC 178
OleNI    IDPDTGCLDPKLVEAAVTPRTGAILGVHLWGRPSRVDELAAIAAEHGLKLFYDAAHALGC 178
GerB     IDPATSCLDPALVEAAITPRTSAIVPVHLWGRPCAVDQLAKVAADHGIKLFYDAAHGLGC 180
DesI     IDPDTGNLDPDQVAAAVTPRTSAVVGVHLWGRPCAADQLRKVADEHGLRLYFDAAHALGC 178

EryCIV   TAGGRPVGAFGNAEVFSFHATKAVTSFEGGAIVTDDGLLADRIRAMHNFGIAPDKLVTDV 238
OleNI    TSRQRRLGSFGDAEVFSFHATKVVNSFEGGGIVTDDDTRAERLRALHNFGLGHDGVG--A 236
GerB     TAEGQPIGGFGQAEVFSFHATKVVNAFEGGAVVTDDEELAQRVRAMHNFGFAQGRVSTET 240
DesI     AVDGRPAGSLGDAEVFSFHATKAVNAFEGGAVVTDDADLAARIRALHNFGFDLPGGSPAG 238

EryCIV   GTNGKMSECAAAMGLTSLDAFAETRVHNRLNHALYSDELRDVRGISVHAFDPGEQNNYQY 298
OleNI    GINAKMSEAAAAMGLTSLEAFADAVASNRANYELYRQELSGLPGVRLIDYDPAERNNYHY 296
GerB     GTNGKMTEAAAAMGLTSLDAFEETVRRNEANYDDYRSELAGIAGLKVMEFNRAERNNYHY 300
DesI     GTNAKMSEAAAAMGLTSLDAFPEVIDRNRRNHAAYREHLADLPGVLVADHDRHGLNNHQY 298

EryCIV   VIISVDSAATGIDRDQLQAILRAEKVVAQPYFSPGCHQMQPYRTEPPLRLENTEQLSDRV 358
OleNI    VIALIDAGVTGLHRDLLLTLLRAENVVAQPYFSPGCHQREPYRTEHPVSLPHTEHLAEQV 356
GerB     LIVEVDAAVTGVHRDLLDGVLRAENVVCQRYFSPGCHEMELYRSERPVTLPHTERLAQRV 360
DesI     VIVEIDEATTGIHRDLVMEVLKAEGVHTRAYFSPGCHELEPYRGQPHAPLPHTERLAARV 358

EryCIV   LALPTGPAVSSEDIRRVCDIIRLAATSGELINAQWDQRTRNGS-------------- 401
OleNI    IALPTGPAVSREDIRRVCDIIRVAAAHGPRITAQAGA-------------------- 393
GerB     LALPTGPTVSREDIRRVCDIVRVTLERGHEVTRRADERVPSTLAP------------ 405
DesI     LSLPTGTAIGDDDIRRVADLLRLCATRGRELTARHRDTAPAPLAAPQTSTPTIGRSR 415
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Figure 1. Multi-alignment analysis of the deduced amino acid sequence of GerB with other known aminotransferases: EryCIV, AAB84075; OleNI, AAD55456; DesI, AAC68684. The conserved amino acids are in bold.

was used for the *S*-adenosylmethionine (SAM)-dependent C-4 deamination rather than for a deoxygenation.[13–15] Further sequence analysis revealed that GerB is similar to DesI (61% identity, Fig. 1), and that GerQ is similar to DesII (73% identity, data not shown). These observations suggest that two genes, GerB and GerQ, are involved in C-4 deoxygenation in the desosamine biosynthetic gene cluster. The GerB protein may be catalyzed by the C-4 transamination of **1** to generate **2** in the dihydrochalocomycin biosynthetic pathway.

2.2. Expression of dTDP-4-keto-6-deoxy-D-glucose aminotransferase, GerB

PCR was performed using the GerB-F and GerB-R primers and the expected PCR product of 1.3-kb was obtained and cloned into the *Bam*HI/*Hin*dIII site of a pET28a expression vector to produce the expression plasmid pGERB. The nucleotide sequence of the cloned *gerB* was confirmed by sequencing. The pGERB plasmid was transformed into *E. coli* BL21 (DE3) and expressed with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) induction to produce a 47-kDa protein comprising GerB (426 aa) (Fig. 2).

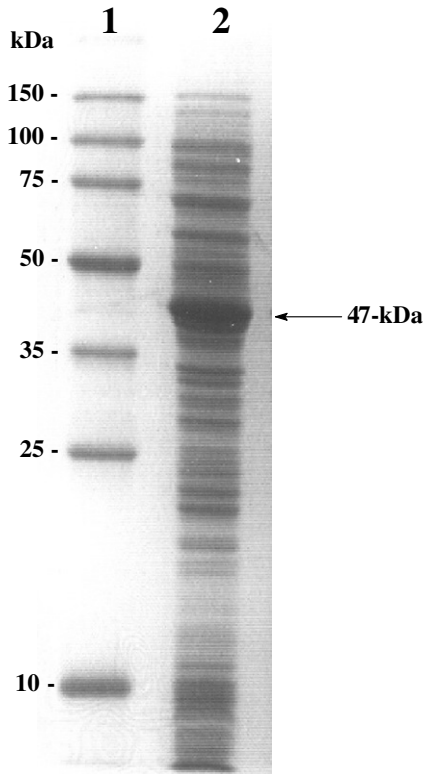


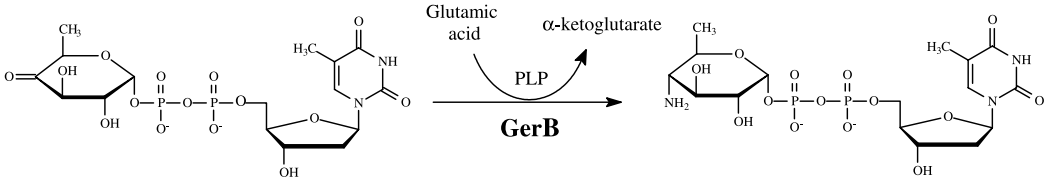
Figure 2. Identification of the over-expressed GerB by SDS–PAGE: lane 1: marker; lane 2: soluble.

2.3. Enzymatic activity of dTDP-4-keto-6-deoxy-D-glucose aminotransferase, GerB

GerB is believed to encode for C-4 transamination in the later stages of D-desosamine biosynthesis. The enzymatic reaction was carried out in 100 mM Tris–HCl buffer (pH 7.5) containing 4 mM **1**, 8 mM L-glutamic acid (or L-glutamine) and 1 mM pyridoxal phosphate (PLP) in order to investigate the activity of GerB. The reaction was initiated by the addition of GerB during incubation at 37 °C. The mixture was screened for the transformation reaction from **1** to **2** using high performance liquid chromatography (HPLC), as shown in Figure 3.[16] The reaction was carried out using L-glutamine or L-glutamic acid as the source of the 4-amino group and L-glutamic acid showed a better conversion to **2** under identical reaction conditions, as clearly shown in the HPLC data (Fig. 4a and b). Thus, L-glutamic acid was used as the source of the amino group for all subsequent reactions. The peak of the expected product (**2**) was observed after the reaction started and included the intermediate peak corresponding to **1**. A conversion yield of roughly 66% was obtained within 5 h.

2.4. One-pot enzymatic synthesis of dTDP-4-amino-4,6-dideoxy-D-glucose

Several nucleotide sugars, such as UDP-D-glucose, dTDP-L-rhamnose and **1**, were effectively synthesized from glucose-1-phosphate through a series of enzymatic reactions in our previous studies.[17–19] The synthesis of **2** was performed using a one-pot reaction (Fig. 5) under the same reaction conditions and starting materials used in previous experiments; TMP, glucose-1-phosphate, L-glutamic acid and PLP. The enzymatic conversion of **2** was achieved using TMK (0.05 mg/mL), ACK (0.04 mg/mL), TGS (0.03 mg/mL), DH (0.03 mg/mL), and dTDP-4-keto-6-deoxy-D-glucose aminotransferase



dTDP-4-keto-6-deoxy-D-glucose (1) **dTDP-4-amino-4,6-dideoxy-D-glucose (2)**

Figure 3. Conversion of dTDP-4-keto-6-deoxy-D-glucose (**1**) to dTDP-4-amino-4,6-dideoxy-D-glucose (**2**) using dTDP-4-keto-6-deoxy-D-glucose aminotransferase (GerB).

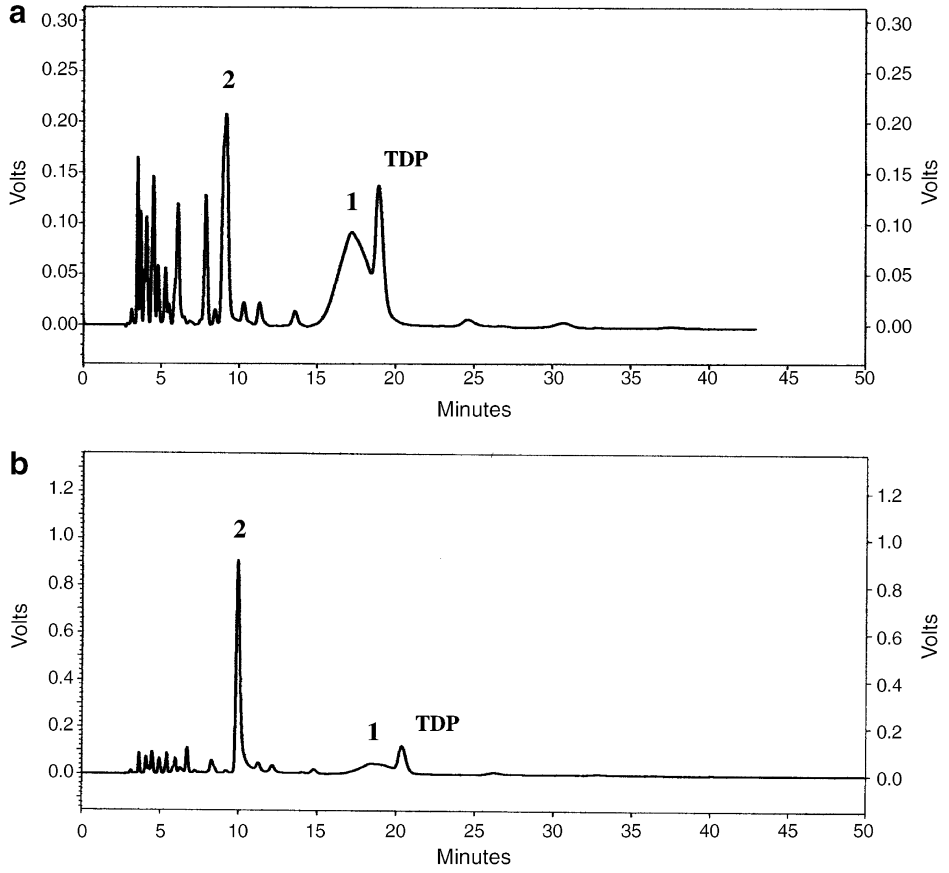


Figure 4. HPLC diagram for the conversion of dTDP-4-keto-6-deoxy-D-glucose (**1**) to dTDP-4-amino-4,6-dideoxy-D-glucose (**2**) by GerB. (a) L-glutamine; (b) L-glutamic acid.

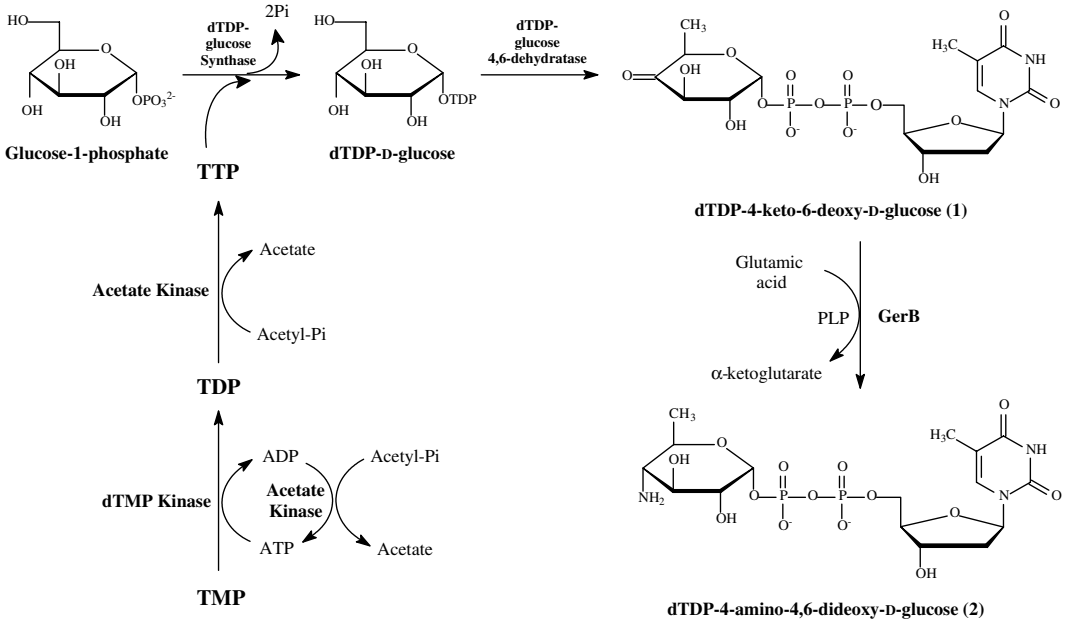


Figure 5. Enzymatic pathway for the one-pot synthesis of dTDP-4-amino-4,6-dideoxy-D-glucose (**2**) from glucose-1-phosphate.

(GerB, 0.04 mg/mL). A conversion yield of approximately 57% was obtained by HPLC peak integration given a reaction time of 270 min. These results confirmed the role of GerB in catalyzing the C-4 transamination of **2** via an amino sugar intermediate in the biosynthesis of D-desosamine.

2.5. Purification and identification of dTDP-4-amino-4,6-dideoxy-D-glucose

Two successive preparative HPLC purifications were performed using mixed solvent systems to isolate **2**. Water and 1.0 M tetraethylammonium borate (TEAB) aqueous solution were used as eluents varying the later concentration from 0% to 80%. Acetonitrile was then used as an eluent and 10 mM TEAB aqueous solution was used to vary the concentration of acetonitrile from 0% to 100%. The purified product was analyzed by analytical HPLC after lyophilization as described in the experimental section. HPLC peak integration proved that **2** was more than 99% pure (Fig. 6). The purified product was subjected to ^{1}H, ^{13}C, and ^{1}H–^{1}H COSY NMR with D_2O rather than $CDCl_3$ as in Bowers et al.'s report.[5] The chemical shifts observed in the proton NMR are closely related to those in the published data with minor shifts down-field. The peak patterns for $H_{3''}$ and $H_{4''}$ were double doublets in the previously reported data; however, those observed in our NMR were triplets since the coupling constants ($J_{2''3''} = 9.9$, $J_{3''4''} = 9.6$, and $J_{4''5''} = 9.8$) are nearly equivalent. The ^{1}H NMR data are summarized in Table 1. Electrospray-MS: m/z 546.3 $[M-H^{+}]^{-}$ calcd for $C_{16}H_{26}N_3O_{14}P_2$: 546.34.

Based on the results of our study, GerB is thought to be a dTDP-4-keto-6-deoxy-D-glucose aminotransferase enzyme responsible for the production of a key interme-

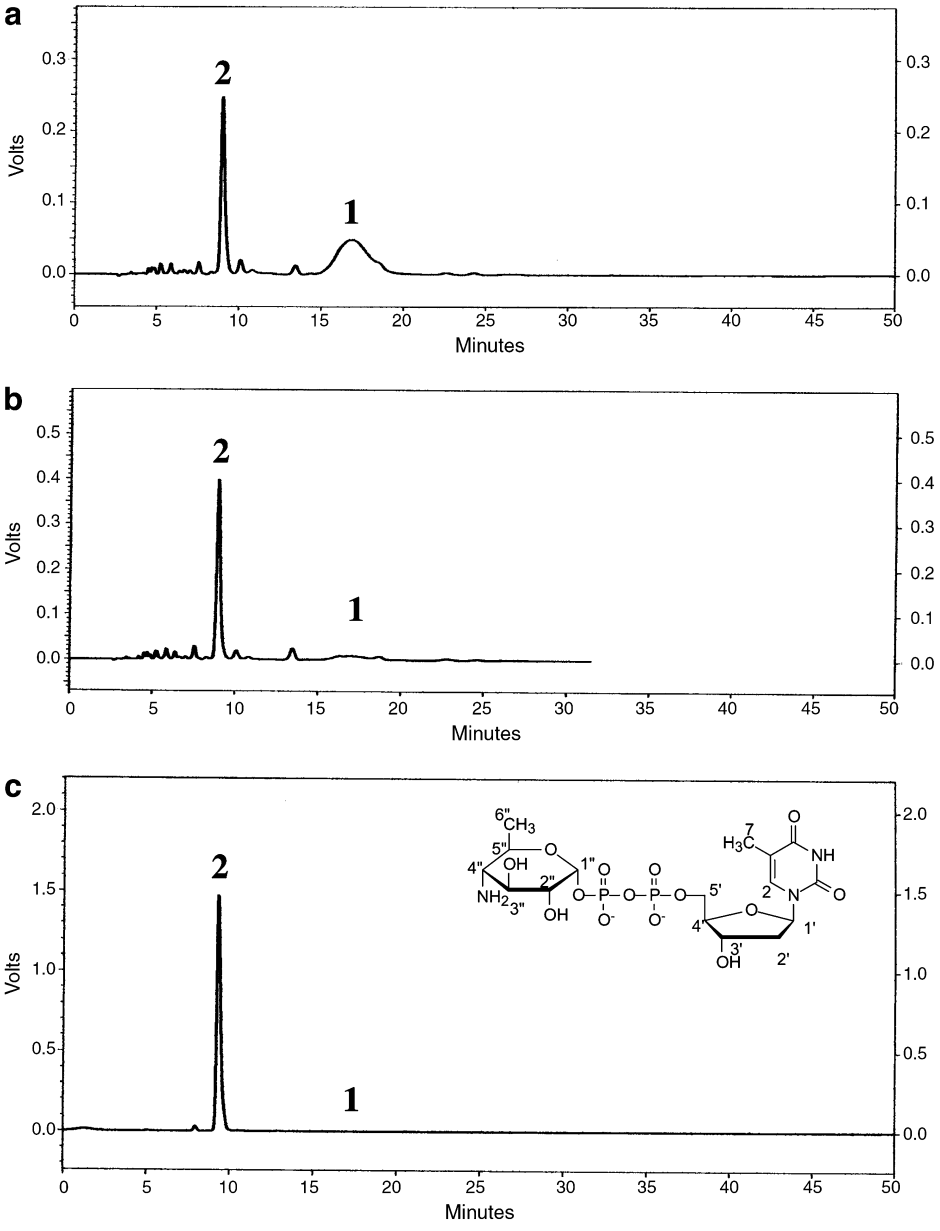


Figure 6. HPLC diagram for the conversion of dTDP-4-keto-6-deoxy-D-glucose (**1**) to dTDP-4-amino-4,6-dideoxy-D-glucose (**2**) using a one-pot reaction. (a) After 150 min; (b) after 270 min; and (c) after two purifications with preparative HPLC.

Table 1. ^{1}H NMR data for the dTDP-4-amino-4,6-dideoxy-D-glucose (**2**) in D_2O

Residue	Proton	Chemical shift (δ, ppm)	Coupling constant (J)
Thymidine	H-2	s, 7.77	
	H-7	s, 1.97	
Ribose	H-1′	t, 6.38	
	H-2′	m, 2.41	
	H-3′	m, 4.66	
	H-4′	m, 4.22	
	H-5′	m, 4.22	
4-Amino-4,6-dideoxy-D-glucose	H-1″	dd, 5.60	J_P 6.9, $J_{1''2''}$ 3.6
	H-2″	dt, 3.60	$J_{2''3''}$ 9.9
	H-3″	t, 3.69	$J_{3''4''}$ 9.6
	H-4″	t, 2.60	$J_{4''5''}$ 9.8
	H-5″	m, 4.02	
	H-6″	d, 1.32	$J_{5''6''}$ 6.0

diate, **2**, in the biosynthesis of deoxyaminosugar (D-desosamine). The results also show that although L-glutamine does show substrate activity in transamination, L-glutamic acid is a better source of the 4-amino group in the conversion to **2**. Moreover, our study indicates that **2** can be synthesized on a preparative scale from glucose-1-phosphate and TMP using five recombinant enzymes without any unknown effects caused by the use of enzymes. This report is the first to describe the preparative enzymatic synthesis of **2**. Therefore, the findings of this study can be used to aid in the synthesis of any other interesting dideoxysugar intermediates including unusual amino sugars based on the generation of an in vitro metabolic pathway.

3. Materials and methods

3.1. Materials

All chemical reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). The dTDP-4-keto-6-deoxy-D-glucose used in this study was provided by the GenChem Co. (Daejeon, Korea). The enzymes used for DNA manipulations in cloning were obtained from Takara (Shiga, Japan). *E. coli* XL1-Blue (MRF) (Stratagene) was utilized as a host cell for the preparation of DNA manipulation, and *E. coli* BL21 (DE3) (Stratagene) was used in the expression of the 6 × His-fusion protein. The pET28a vector was purchased from Novagen (West Palm Beach, FL) for use in gene expression. The cloning and transformation techniques used in this study were adapted from those described by Sambrook et al.[20]

3.2. Construction of the expression vector

GerB from *S.* sp. Geri-155 was cloned into the expression vector using the polymerase chain reaction. A set of modified primers harboring the GerB-F (5′-GAA GGA TCC ACC ATG AAA CGC AAT GTC-3′) and the reverse GerB-R (5′-CCG CAA GCT TTT CCC TGA TCA GGG GGC-3′) sequences were designed for PCR amplification of the GerB coding region (the restriction sites, *Bam*HI and *Hin*dIII, are indicated by underlined letters). PCR was conducted under the following conditions: 1 cycle for 7 min at 97 °C; 30 cycles for 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C; 1 cycle for 7 min at 72 °C. The PCR products of the expected size (1.2-kb) were cloned into the *Bam*HI/*Hin*dIII site of the pET28a expression vector to generate the expression plasmid pGERB.

3.3. Expression of GerB

E. coli BL21 (DE3) cells harboring pGERB were grown aerobically in 50 mL of Luria broth (LB) using a 250 mL shaking flask at 37 °C with agitation at 200 rpm until an OD_{600} of 0.6 was obtained and was followed by the addition of 1 mM IPTG for induction in order to express GerB. The cultures were then harvested, and pelleted after incubation for 18 h at 20 °C. Crude extracts were obtained by sonicating in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM (DTT), 10% glycerol, 1 mM (EDTA), 2 mM $MgCl_2 \cdot 6H_2O$ and 1 mM (PMSF). The lysate was then centrifuged at 15,000*g* for 30 min at 4 °C; the precipitate was discarded.

3.4. Enzyme assay and enzymatic synthesis

GerB was assayed using 4 mM **1**, 8 mM L-glutamic acid (or L-glutamine), 1 mM PLP and 100 mM Tris–HCl (pH 7.5). The activities of GerB were identified by comparing the conversion of **1** to **2**. The in vitro enzymatic reactions were conducted in 200 mL of reaction mixture containing 10 mM TMP, 0.25 mM ATP, 20 mM $MgCl_2$, 50 mM acetyl-phosphate, 40 mM glucose-1-phosphate, 20 mM L-glutamic acid, 0.2 mM PLP, 50 mM Tris–HCl (pH 7.5) and enzyme extracts in order to accomplish a one-pot enzymatic synthesis. All other enzyme activities were determined in 50 mM Tris–HCl buffer (pH 7.5) at 37 °C. The activities of TMK, ACK, TGS, and DH were measured as previously described.[17] The reaction was initiated with the addition of approximately 20 mL of TMK (0.05 mg/mL), 20 mL of ACK (0.04 mg/mL), 20 mL of TGS (0.03 mg/mL), 20 mL of DH (0.03 mg/mL), and 20 mL of dTDP-4-keto-6-deoxy-D-glucose aminotransferase (GerB, 0.04 mg/mL). The reaction was carried out for 270 min at 37 °C and was terminated upon heating the reaction mixture at 100 °C for 1 min. The reaction mixture was diluted with the same buffer (1:5), and the reactions were monitored by HPLC. The final product was purified using SHIMADZU SPD-10Avp (source Q15 resin 200 mL, FineLine Pilot 35 column, Amersham biosciences) and an ODS-AP 300-S10/20 $30\Phi \times 500$ mm

column (Kyoto). The lyophilized product was characterized with ^{1}H NMR, ^{1}H COSY, ^{13}C NMR, and ESI-MS, and its purity was analyzed by HPLC.

3.5. Instrumental analysis

Various nucleotides and **2** were analyzed by HPLC using a strong anion exchange column (Hypersil ODS 4.6×250 mm, 5 μm particle size) with 100 mM potassium phosphate buffer (pH 7.0):methanol = 95:5 (v/v). The eluted nucleotides were monitored by absorbance at 270 nm at a flow rate of 1.0 mL/min. ESI mass spectrometry analysis was performed in negative ion mode at a voltage of 5 V using a Thermo Finnigan AQA mass spectrometer (Thermo Finnigan) equipped with the standard Thermo ESI source. The purified products were dissolved in 0.70 mL D_2O (99.9 atom % D) and used for NMR analysis in order to identify the final product. ^{1}H, ^{1}H COSY and ^{13}C NMR spectra were recorded using a Varian UNITY-INOVA 300 MHz NMR spectrometer (Varian) at a probe temperature of 298 K, and the chemical shifts were expressed in ppm relative to the internal HOD signal (4.80).

Acknowledgement

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