Biosynthesis of dTDP-6-deoxy-β-D-allose, biochemical characterization of dTDP-4-keto-6-deoxyglucose reductase (GerK1) from Streptomyces sp. KCTC 0041BP

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dTDP-6-deoxy-D-allose, an unusual deoxysugar, has been identified as an intermediate in the mycinose biosynthetic pathway of several macrolide antibiotics. In order to characterize the biosynthesis of this deoxysugar, we have cloned and heterologously overexpressed gerK1 in Escherichia coli BL21 (DE3) cells. This gene encodes for a protein with the putative function of a dTDP-4-keto-6-deoxyhexose reductase, which appears to be involved in the dihydrochalcomycin (GERI-155) biosynthesis evidenced by Streptomyces sp KCTC 0041BP. Our results revealed that GerK1 exhibited a specific reductive effect on the 4-keto carbon of dTDP-4-keto-6-deoxy-D-allose, with the hydroxyl group in an axial configuration at the C3 position only. The enzyme catalyzed the conversion of dTDP-4-keto-6-deoxyglucose to dTDP-6-deoxy-β-D-allose, according to the results of an in vitro coupled enzyme assay, in the presence of GerF (dTDP-4-keto-6-deoxyglucose 3-epimerase). The product was isolated, and its stereochemistry was determined via nuclear magnetic resonance analysis.

Keywords: deoxysugar biosynthesis/dTDP-6-deoxy-β-D-allose/dTDP-4-keto-6-deoxyglucose reductase/Streptomyces

Introduction

Deoxysugars, all of which harbor certain hydroxyl groups that have been replaced by a hydrogen atom, an amino group, and an alkyl side chain, are vital constituents of the aglycon core of several bacterial antibiotics and anticancer drugs. Functionally, these deoxysugars participate in the recognition and interaction of bioactive compounds with drug targets (Weymouth- wilson 1997; Hecht 1999; Hansen et al. 2002). Therefore, they play a crucial role in the optimal biological antibiotic activity of bioactive compounds, and the removal of these moieties has been shown to strongly influence or completely abolish the antibacterial activity of certain compounds (Rohr 1997). Among the unusual sugars, dTDP-6-deoxy-D-allose has been identified as a crucial intermediate in the mycinose biosynthetic pathway of several macrolide antibiotics, including dihydrochalcomycin (GERI-155 used in this study; Jaishy et al. 2006), tylosin (Fouces et al. 1999), chalcomycin (Ward et al. 2004), and mycinamicin II (Anzai et al. 2003). Structurally, this sugar attaches to the macrolactone by the glycosidic linkage with the activity of the glycosyltransferase (Wilson and Cundliffe 1998) and requires a primary hydroxyl group at C20 of the macrolactone with the activity of a P450 enzyme (Fouces et al. 1999; Ward et al. 2004). The biosynthetic pathway of dTDP-6-deoxy-D-allose is presumably comprised of several enzyme reaction steps. It begins with the nucleotidyl activation of glucose-1-phosphate to form dTDP-D-glucose by the activity of dTDP-D-glucose synthase, GerD (Lee, Sohng, Kim, Nam, Han et al. 2004), and TylA1 (Merson-Davies and Cundliffe 1994). Catalysis by dTDP-D-glucose-4,6-dehydratase, GerE (Lee, Sohng, Kim, Nam, Seong et al. 2004), and TylA2 (Merson-Davies and Cundliffe 1994) results in the formation of dTDP-4-keto-6-deoxy-D-glucose, a common intermediate in the biosynthetic pathways of the majority of the deoxysugars derived from dTDP-D-glucose (Rodriguez et al. 2002; Lombo et al. 2004; Maki and Renkonen 2004). Finally, the reduction of the 4-carbonyl carbon by 4-ketoreductase TylD (Bate and Cundliffe 1999; Fouces et al. 1999) (GerK1 in this study) should occur after the 3-epimerization of dTDP-4-keto-6-deoxy-D-glucose by dTDP-4-keto-6-deoxy-D-glucose 3-epimerase GerF (Sohng et al. 2004) (Figure 1B). We have recently isolated the mycinose gene cluster involved in the dihydrochalcomycin biosynthesis from Streptomyces sp. KCTC 0041BP (GenBank accession no. AY118081). Six open reading frames (ORFs) (gerE, gerD, gerF, gerK1, gerM2, and gerM3) were predicted to be mycinose biosynthetic genes. Out of these six ORFs, gerK1 was suggested to encode for dTDP-4-keto-6-deoxyhexose reductase and was thought to be involved in the biosynthesis of dTDP-6-deoxy-D-allose.

The dTDP-4-keto-6-deoxyhexose reductases are very important enzymes and are found in the abundant deoxysugar gene clusters of secondary metabolites. These enzymes have been demonstrated to catalyze a reduction at the C4 keto of activated sugars with different stereochemistry, thereby...
creating a number of unusual sugars. The *gerK1* encodes for NDP-4-keto-6-deoxyhexose reductase, which acts on the 4-keto carbon of the 4-keto-6-deoxy-D-hexose with the axial OH at C3 only. However, no reports have yet been published regarding the biosynthesis of dTDP-6-deoxy-D-allose, either in in vivo or in in vitro systems and the functions of this compound have yet to be definitively verified. The primary objective of this study was to determine the functions of *gerK1* in the mycinose biosynthetic pathway and to isolate the enzymatic product for a determination of its stereochemistry via nuclear magnetic resonance (NMR) analysis. We therefore cloned and heterologously expressed this gene in *Escherichia coli* BL21 (DE3) and conducted in vitro assays using the expressed protein.
Nucleotide sequence analyses

ORF gerK1 (0.98 kb) is located within the deoxysugar gene cluster associated with the dihydrochalcomycin biosynthesis, flanked downstream by gerL and upstream by gerM (Figure 1A). Using the Clustal X program, the determined sequence analysis of the GERI-155 gene cluster revealed a deduced amino acid sequence (327 amino acids) encoded for GerK1, which displays a very high degree of similarity to a number of the known dTDP-4-keto-6-deoxyhexose reductases in the GenBank database (Figure 2), including ChmD (89% identity) (GenBank accession no. AAS79455) (Ward et al. 2004), MydI (56% identity) (GenBank accession no. BAC57025) (Anzai et al. 2003), and TylD (52% identity) (GenBank accession no. AAD41816) (Fouces et al. 1999). The primary structure of GerK1, in comparison with other members of the ketoreductase family, displays a highly conserved region for two motifs of the binding sites. The first motif, (16\text{G}XX\text{G}XX\text{G}22), is located in the vicinity of an N-terminal region, which has been identified as a nicotinamide adenine dinucleotide (NADH) cofactor-binding motif in which glycines are the active residues that bind to the cofactor (Aguirrezabalaga et al. 2000; Giraud and Naismith 2000; Allard et al. 2001). The second motif is located at the C-terminal that harbors the catalytic triad of (16\text{Y}XXX\text{K} XXEx), which is found almost exclusively in the reductase/epimerase/dehydrogenase (RED) homology super family, in which tyrosine and lysine constitute the key residues for the catalytic activity and substrate binding of this enzyme (Giraud et al. 1999; Dong et al. 2003; Watt et al. 2004).

Expression and purification of GerK1 and GerF

E. coli BL21 (DE3) cells were transformed with the pGerK1 and pGerF expression plasmids for the expression of gerK1 and gerF with the 6\text{His}/C2 histidine and thioredoxin-tagged fusion protein, respectively. The induction of the expression of the transformants harboring pGerK1 and pGerF plasmids with isopropyl-\text{b-D-thiogalactopyranoside} (IPTG) at 20 °C for 20 h resulted in an excessive formation of soluble proteins. The soluble fractions of GerK1 and GerF were then purified via Ni\text{2+} affinity chromatography, as outlined in the Materials and methods section. The molecular weight of the denatured protein analyses was consistent with the calculated value of 35.9 kDa as molecular weight of GerK1 and 21.6 kDa as molecular weight of GerF, translated from peptide sequences. The bands observed via SDS–PAGE at approximately 52 and 38 kDa (Figure 3), including the 6\text{His}-tag and thioredoxin-tagged fusion protein, correspond to the dominant expression products of gerK and gerF, respectively. These target proteins were almost completely eluted by 100 mM imidazole in the sodium phosphate buffer solution during the purification process. The concentrations of the purified GerK1 and GerF were measured as 0.06 and 0.004 mg/mL.

Characterization, function of GerK1 and biosynthesis of dTDP-6-deoxy-\text{b-D-allose}

In order to determine the predicted function of GerK1, a coupled enzyme reaction (reaction I) was conducted with the GerK1 and GerF enzymes, which were incubated with dTDP-4-keto-6-deoxy-\text{D-glucose} in the presence of NADH and MgCl2 as cofactors, in accordance with the protocol described in the Materials and methods section. The function
of GerF was characterized as dTDP-4-keto-6-deoxyglucose 3-epimerase (Sohng et al. 2004). The appropriate quantity of quenched reaction mixture was then withdrawn and subjected to high-pressure liquid chromatography (HPLC) analysis. Interestingly, both the oxidation of NADH and the reduction of dTDP-4-keto-6-deoxyglucose were observed by remarkable decreasing of substrate peak with a 10-min retention time (Figure 4A), and a new peak was detected on HPLC with a 13-min retention time, as the dominant product (Figure 4B). The results of enzymatic conversion clearly indicated that this ketoreduction took place in the presence of GerK1 and GerF. Similarly, the electrospray ionization mass spectrometry (ESI-MS) analysis revealed that the appearance of a peak corresponding to \( m/z \) 547 (M + H\(^+\)) indicated a very high conversion rate, whereas the peak corresponding to \( m/z \) 545 (M + H\(^+\)) of dTDP-4-keto-6-deoxyglucose was attenuated markedly. This result further confirmed the formation of a reduced substrate and also bolstered the notion that GerK1 functions as a dTDP-4-keto-6-deoxyglycose reductase in the pathway of dTDP-6-deoxy-D-allose biosynthesis.

In order to ascertain whether GerK1 might act as a flexible enzyme, reacting with different stereochemical substrates, the enzyme was incubated with dTDP-4-keto-6-deoxyglucose in the presence of NADH and MgCl\(_2\) as cofactors (reaction II). No NADH oxidation was observed on UV analyses, nor was the reduction of the substrate. No peaks appeared with the 13-min retention time on HPLC and/or the peaks corresponding to \( m/z \) 547 (M + H\(^+\)) on the ESI-MS analyses, in contrast with what was observed in reaction I. These findings clearly indicated that GerK1 reacts with substrate only in the presence of GerF, or that ketoreduction occurs after the 3-epimerization step inherent to the biosynthesis of dTDP-6-deoxy-D-allose.

The subsequent large-scale incubation of the reaction mixture allowed for the isolation and purification of a new nucleotide sugar. This enzymatic reaction was performed in the same molar concentration of NADH, and HPLC analysis showed a complete conversion of substrate within 3 h of incubation. Typically, a very broad substrate peak appeared at a retention time of 10 min, whereas the product peak appeared at a retention time of 13 min. The isolation of the enzymatic product begins with the removal of proteins via the heating and subsequent centrifugation of the reaction mixture. The obtained supernatant then undergoes two chromatographic steps, as described in the Materials and methods section. The final yield was subjected to HPLC analysis.

**Analysis of dTDP-6-deoxy-β-D-allose by NMR**

The purified dTDP-6-deoxy-D-allose was subjected to NMR analysis. The full \(^1\)H-NMR spectra revealed signals indicating the dTDP-6-deoxy-β-D-allose compound. The stereochemistry of the sugar compound was assigned on the basis of correlation spectroscopy analysis. The cross peaks from H\(_1\), H\(_2\), H\(_3\), and H\(_4\) locate the H\(_1\) at 5.38 ppm, the H\(_2\) at 3.56 ppm, the H\(_3\) at 3.95 ppm, the H\(_4\) at 3.25 ppm, the H\(_5\) at 4.02 ppm, and the H\(_6\) at 1.13 ppm. The small \( J_{1',2'} \) coupling constant (3.75 Hz) and the small \( J_{2',3'} \) coupling constant (3.6 Hz) of the pyranose moiety in the NMR spectrum indicate that this sugar harbors equatorially disposed H\(_1\) and H\(_2\) protons and also possesses an equatorially disposed H\(_3\) proton. Similarly, the small \( J_{3',4'} \) coupling constant (3.2 Hz) and the large \( J_{4',5'} \) coupling constant (10.1 Hz) of the sugar moiety reveal that the compound possesses an equatorially disposed H\(_4\) and an axially disposed H\(_5\) proton (Hecht 1999). The hydroxyl group at C4 has been
suggested to be equatorially disposed, and therefore, the compound is considered to be in \( \alpha \) configuration.

**Discussion**

The biochemical characterization of GerK1, which is involved in the pathways of dTDP-6-deoxy-\( \beta \)-\( \alpha \)-allose biosynthesis, was addressed via the performance of an in vitro enzyme assay, which employed dTDP-4-keto-6-deoxyglucose as the substrate. This enzyme utilizes NADH to reduce the 4-carbonyl carbon group of dTDP-4-keto-6-deoxyglucose, resulting in the formation of a hydroxyl group at C4 of the expected product. With regard to mechanics, GerK1 transfers protons with stereospecificity from the NADH to the C4 of substrate, resulting in the production of dTDP-6-deoxy-\( \alpha \)-allose.

NDP-4-keto-6-deoxyhexose reductase is a member of the ketoreductase family. The enzyme has been characterized to involve in biosynthesis of several unusual sugars of bacterial strains and has been used as a very efficient tool for the creation of numerous intermediate sugars. This family of enzymes is known to employ different NDP-4-keto-6-deoxyhexoses as precursor substrates and it can be classified into two groups (Figure 5). Group I of the 4-ketoreductases belongs to the RED superfamily (as mentioned in Nucleotide sequence analyses) and has been determined to accept NDP-4-keto-6-deoxyglucose as a precursor substrate. Enzymes act on the C4 carbonyl carbon, following different enzyme reaction steps within the special pathways, thereby resulting in the formation of a number of the activated deoxysugars. Ketoreduction occurs after the 3,5-epimerization step, resulting in the formation of dTDP-\( \alpha \)-rhamnose (Giraud and Naismith 2000; Madduri et al. 2001), and 2,3-dehydration and 3-ketoreduction result in the formation of dTDP-olivose (Aguirrezabalaga et al. 2000). In other sugar biosynthesis pathways, 4-ketoreduction occurs after 3,5-epimerization and methylation, resulting in the formation of dTDP-\( \alpha \)-noviose (Thuy et al. 2005), and 2,3-dehydration, 3-ketoreduction, and C-methylation result in the formation of mycarose (Takahashi, Liu, Chen et al. 2005). Some of the 4-ketoreductases in this group exhibit flexible activity and might be capable of accepting other 4-ketosugar substrates. For example, the EryBIV ketoreductase has been shown to reduce the 4-keto group at C4 on both C3-methylated and unmethylated intermediates, with different stereochemistry with regard to the hydroxyl group at the C3 position (Rodriguez et al. 2002). The 4-ketoreductases in group II have been identified as members of the short-chain dehydrogenase/reductase (SDR) protein family, a branch of the aforementioned RED superfamily. The SDR family was identified in bacterial enzymes, using GDP-4-keto-6-deoxy-\( \alpha \)-mannose as a precursor substrate. The corresponding crystal structures are known to harbor the coenzyme-binding pattern, TGXXGXXG, and the active site pattern, S-YXXXK (Maki and Renkonen 2004), in which serine, tyrosine, and lysine function as active site residues, as also occurs in the RED family (the active residues are indicated in boldface).

With regard to mechanics, GerK1 transfers protons with stereospecificity from the NADH to the C4 of substrate, resulting in the formation of a hydroxyl group at C4 of the expected product. This enzyme utilizes NADH to reduce the 4-carbonyl carbon of the nucleotide sugar, with the hydroxyl group at the C3 position in the axial configuration only. According to the findings of a previous study, GerF exhibits epimerase activity, as evidenced by the detection of maltol, the decomposition product of the activated intermediate (Sohng et al. 2004). The confirmation of GerF as a 3-epimerase or a 3,5-epimerase has yet to be completed owing to failures to acquire an isolation product from the GerF enzyme assay. However, the successful isolation of dTDP-6-deoxy-\( \alpha \)-allose provided evidence sufficient to confirm that GerF functions as a dTDP-4-keto-6-deoxyglucose 3-epimerase in the mycinose biosynthesis pathway (Figure 6).

The present study concerning GerK1 allowed us to examine possible alternate synthetic routes, employing dTDP-6-deoxy-\( \beta \)-\( \alpha \)-allose, an intermediate nucleotide sugar that is synthesized
continuously in metabolite biosynthesis from dTDP-4-keto-6-deoxyglucose, as mentioned in the Introduction section and Figure 1B. It has also provided an efficient tool for the large-scale biosynthesis via the combination of GerK1 with the recombinant GerD (Lee, Sohng, Kim, Nam, Han et al. 2004), GerE (Lee, Sohng, Kim, Nam, Seong et al. 2004), and GerF (Sohng et al. 2004) present in the mycinose biosynthetic gene cluster in *Streptomyces* sp. KCTC 0414BP. The recombinant GerK1 and GerE have yet to be completed. However, dTDP-6-deoxy-D-allose is expected to be a viable substrate for the in vitro characterization of the sugar genes (gerMII- and gerMIII-encoding O-methyltransferases) functioning in the penultimate steps of the mycinose biosynthetic pathway. This compound is also known to be an ideal glycone donor for the determination of the glycosyltransferases encoded for by the gerT1 or gerT2 involved in the GERI-155 biosynthetic pathway.

### Materials and methods

**Chemicals and reagents**

The dTDP-4-keto-6-deoxy-D-glucose used in this study was provided by the Genchem Co. (Daejeon, Korea), and the NADH and IPTG were purchased from the Sigma Chemical Co. (St. Louis, MO). All restriction enzymes used in cloning were obtained from the Takara Co. (Shiga, Japan). Polymerase chain reaction (PCR) was carried out by using a Pre-Mix™—Top kit (Bioneer, Korea). The other chemicals were high-grade products obtained from commercially available sources.

**Bacteria growth condition and vectors**

*E. coli* XL1-Blue (MRF) (Stratagene, La Jolla, CA) was used as a host cell for the preparation of the recombinant plasmids and DNA manipulation, whereas *E. coli* BL21 (DE3) (Stratagene) was used as a host for the expression of the 6× his-fusion protein. *E. coli* was grown at 37°C in Luria-Bertani (LB) broth or on an agar plate supplemented with the appropriate amount of antibiotics whenever necessary (ampicillin up to 100 μg/mL) for the selection or maintenance of the plasmids, pGEM-T easy system I (Promega, Madison, WI) was used as a vector for the cloning of the PCR products, and pET-32a(+) (Novagen, Madison, WI) was employed in the gene expressions. The pGER5 cosmid (42,147 bp) (GenBank accession no. Y118081) harboring the deoxy sugar gene cluster was used as a template for DNA amplification.

**Gene manipulation and construction of expression vectors**

DNA preparation, its manipulations, restriction endonuclease digestion, and DNA ligation were all conducted in accordance with the standard protocols (Sambrook et al. 1989). Computer-aided database searches and sequence analyses were carried out using the BLAST server (http://www.ncbi.nlm.nih.gov) (Altschul et al. 1990) and the Clustal X program. A set of primers harboring the gerK1-F 5′-CC AAG CTT GGA TCC GCA GGA CAC AGA GTG A-3′ and the gerK1-R 5′-GG

GAA TTC TAG AGG GGT TCA GCG CTT GT-3′ sequences were employed in the amplification of gerK1 (the restriction sites are shown in boldface). The PCR products (0.99 kb) were cloned into pGEM-T easy vector (Promega) and sequenced prior to cloning into the expression vector in order to ensure that no mutations were introduced during PCR amplification. The PCR was conducted in a thermocycler (Takara) under the following conditions: 25 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C. The PCR product was then cloned into the BamHI/EcoRI site of pET-32a(+) to generate pGerK1 as the expression plasmid in the *E. coli* BL21 (DE3) host. dTDP-6-deoxyglucose 3-epimerase (GerF) was expressed by using the plasmid pHJ3 (Sohng et al. 2004).

**Expression and enzyme purification**

For the expression of gerK1 and gerF, *E. coli* BL21 (DE3) cells harboring pGerK1 and pGerF were grown separately in 10 mL of LB medium supplemented with ampicillin at 37°C, with agitation at 250 rpm for 8 h. The cultures were then transferred to 600 mL of fresh LB medium, incubated at 37°C, and shaken at 250 rpm. At an OD_{600} of 0.6, IPTG was added to a final concentration of 0.4 mM, and incubation continued for 20 h at 20°C. The cells were then harvested via centrifugation for 10 min, washed twice in cold condition at 4°C with sodium phosphate buffer (50 mM, pH 7.5), resuspended in 12 mL of the same buffer, and finally stored at −20°C for 6 h.

For the purification of GerK1 and GerF, the cell pellets were suspended in 12 mL of sodium phosphate buffer (50 mM, pH 7.5). The cell pellets were then disrupted via ultrasonification, and the crude extracts were acquired by centrifugation at 12 000 rpm. The enzymes were purified via Ni^{2+}-affinity chromatography (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instruction. The proteins were eluted with a linear gradient of imidazole (20–150 mM) solution in the aforementioned phosphate buffer. The pure fractions were then dialyzed with a storage buffer (50 mM sodium phosphate buffer, pH 7.5) for 8 h at 4°C. The protein concentrations were determined in accordance with the Bradford assay procedures (Bradford 1976).

**Enzyme assay of GerK1 and isolation of dTDP-6-deoxy-D-allose**

In order to determine the activity of GerK1, in vitro-coupled enzyme reactions were conducted in 100 μL of reaction mixture, following the previously described protocol (Chen et al. 2000), with slight modifications. The reaction mixture was comprised of 50 mM sodium phosphate buffer (pH 7.5), 4 mM dTDP-4-keto-6-deoxy-D-glucose, 8 mM NADH, and 1 mM MgCl_{2}. The reaction was initiated with the addition of around 30 μL of GerK1 (0.06 mg/mL) and 30 μL of GerF (0.004 mg/mL). The mixture was incubated for 2 h at 37°C and finally quenched by 1 min of heating at 70°C. The precipitated proteins were then removed via 10 min of centrifugation at 12 000 rpm, after which the supernatant was withdrawn and subjected to HPLC and ESI-MS analyses. The HPLC analyses were conducted using an isocratic elution program in a solvent system containing 100 mM potassium phosphate buffer (pH 7.0) and methanol (95:5) at a flow rate of 1 mL/min, using a C-18 column (C-18 X Terra™ RP18, 5 μm 4.6 × 250 mm, Waters, Ireland).
The NMR spectra were measured using a VARIAN-INOVA 400-MHz spectrometer and collected in D$_2$O. The NMR spectra were measured using a VARIAN-INOVA 400-MHz spectrometer and collected in D$_2$O. The NMR spectra were measured using a VARIAN-INOVA 400-MHz spectrometer and collected in D$_2$O. The NMR spectra were measured using a VARIAN-INOVA 400-MHz spectrometer and collected in D$_2$O.

The purified compound was subjected to ESI-MS and NMR analysis. ESI-MS, electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; IPTG, isopropyl-$\beta$-D-thiogalactopyranoside; LB, Luria-Bertani; NADH, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; ORFs, open reading frames; PCR, polymerase chain reaction; RED, reductase/epimerase/dehydrogenase; SDR, short-chain dehydrogenase/reductase.

Table I. NMR spectroscopic identification of dTDP-6-deoxy-$\beta$-D-allolose

<table>
<thead>
<tr>
<th>Residue</th>
<th>Proton</th>
<th>Chemical shifts ({$\delta$}, ppm)</th>
<th>Coupling constant ($J$)</th>
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<td></td>
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<td></td>
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<td></td>
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The subsequent large-scale incubation of reaction mixture allowed us to isolate the products. The enzymatic reaction was conducted with an equimolar concentration of NADH in 100 mM sodium phosphate buffer, and the complete conversion of substrate to product within 3 h of incubation was demonstrated by HPLC analysis. After the complete conversion, as determined by HPLC analysis, the crude extract contained product was taken out from the quenched enzyme assay mixture by the same method as mentioned above, and the compound was purified via preparatory HPLC with a C-18 (SHISEIDO) Prep HPLC–RP column (CAPCELL PAK C-18 10 $\times$ 250 mm). Purification was conducted using the isotatic elution program in sodium phosphate buffer of 100 mM, and elution was monitored at 270 nm. At the appearance of the expected peak at the accurate retention time, the fraction containing the product was collected. Finally, sodium salts were removed from the compound via the application of the sample to a 1.6 $\times$ 70 cm Sephadex G-10 column in H$_2$O as mobile phase, after which the compound was dried under deep-freeze conditions, using a freeze dryer.

**NMR and ESI-MS analysis**

The purified compound was subjected to ESI-MS and NMR analyses. The mass spectra for C$_{16}$H$_{26}$N$_{20}$O$_{15}$P$_{22}$ was calculated to be 548.33 and was observed to be 547 (M – H$^-$). The NMR spectra were measured using a VARIAN-INOVA 400-MHz spectrometer and collected in D$_2$O. The NMR results are shown in Table I. Funding to pay the Open Access publication charges for this article was provided by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant MG02-0301-004-2-3-1).

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**Conflict of interest statement**

None declared.

**Abbreviations**

ESI-MS, electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; IPTG, isopropyl-$\beta$-D-thiogalactopyranoside; LB, Luria-Bertani; NADH, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; ORFs, open reading frames; PCR, polymerase chain reaction; RED, reductase/epimerase/dehydrogenase; SDR, short-chain dehydrogenase/reductase.

**References**


Alam J, Beyer N, Liu HW. 2004. Biosynthesis of colistoside: expression, purification, and mechanistic characterization of GDP-4-keto-6-deoxy-$\alpha$-mannose-3-dehydratase (ColD) and GDP-$\alpha$-colistose synthase (ColC). Biochemistry. 43:16450–16460.


