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ORIGINAL RESEARCH PAPER

Cloning and characterization of CalS7 from *Micromonospora echinospora* sp. *calichensis* as a glucose-1-phosphate nucleotidyltransferase

Dinesh Simkhada · Tae-Jin Oh · Eui Min Kim · Jin Cheol Yoo · Jae Kyung Sohng

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Abstract The deoxysugar biosynthetic gene cluster of calicheamicin contains the *calS7*, which encodes glucose-1-phosphate nucleotidyltransferase and converts glucose-1-phosphate and nucleotides (NTP) to NDP-glucose and pyrophosphate. *calS7* was expressed in *Escherichia coli* BL21(DE3), and the purified protein had significant thymidylyltransferase and uridylyltransferase activities as well, with some guanidylyltransferase activity but negligible cytidyl and adenyltransferase activity. The functions of thymidylyltransferase and uridylyltransferase were also verified using one-pot enzymatic synthesis of TMK and ACK. The products were analyzed by HPLC and ESI/MS, which showed peaks at $m/z = 563$ and 565 for TDP-D-glucose and UDP-D-glucose, respectively, in negative mode.

Keywords Calicheamicin · Deoxysugar · *Micromonospora echinospora* sp. *calichensis* · Thymidylyltransferase · Uridylyltransferase

D. Simkhada · T.-J. Oh · E. M. Kim · J. K. Sohng (✉)
Institute of Biomolecule Reconstruction (iBR), Department of Pharmaceutical Engineering, Sun Moon University, # 100, Kalsan-ri, Tangjeong-myeon, Asansi, Chungnam 336-708, Republic of Korea
e-mail: sohng@sunmoon.ac.kr

J. C. Yoo
Department of Pharmacy, College of Pharmacy, Chosun University, Gwangju 501-759, Korea

Introduction

Calicheamicin produced by *Micromonospora echinospora* ssp. *calichensis* is an endiyne antibiotic containing three distinct structural elements: a DNA-recognition unit, which delivers the metabolite to its target DNA; an activation component, which sets the stage for cycloaromatization; and the enediyne "warhead", which cycloaromatizes to a highly reactive diradical species in the presence of DNA (Myers et al. 1994; Biggins et al. 2003) (Fig. 1). The basic property of calicheamicin resides is its ability to cleave DNA. This cleavage property can be triggered by the action of a thiol-reducing agent or UV exposure. The enediyne moiety plays an important role in DNA cleavage, while the sugar parts are supposed to help the antibiotic become more specific or direct it to bind the double-stranded DNA. In this sense, the all-structural constituents are equally important (Biggins et al. 2003; Galm et al. 2005).

The nucleotide-sugar NDP-D-glucose is a key metabolite in prokaryotes where it serves as a precursor to a large number of modified sugars, such as L-rhamnose (6-deoxyhexose), 6-deoxy-L-talose, 2,6-dideoxyhexose and other deoxyhexoses and deoxypentose (Simone et al. 2001). Numerous studies have demonstrated that the early enzymatic step common to the biosynthesis of all deoxysugars found in antibiotics is the formation of NDP-glucose from NTP and α-D-glucose-1-phosphate from glucose-1-phosphate nucleotidylyltransferase (NDP-glucose

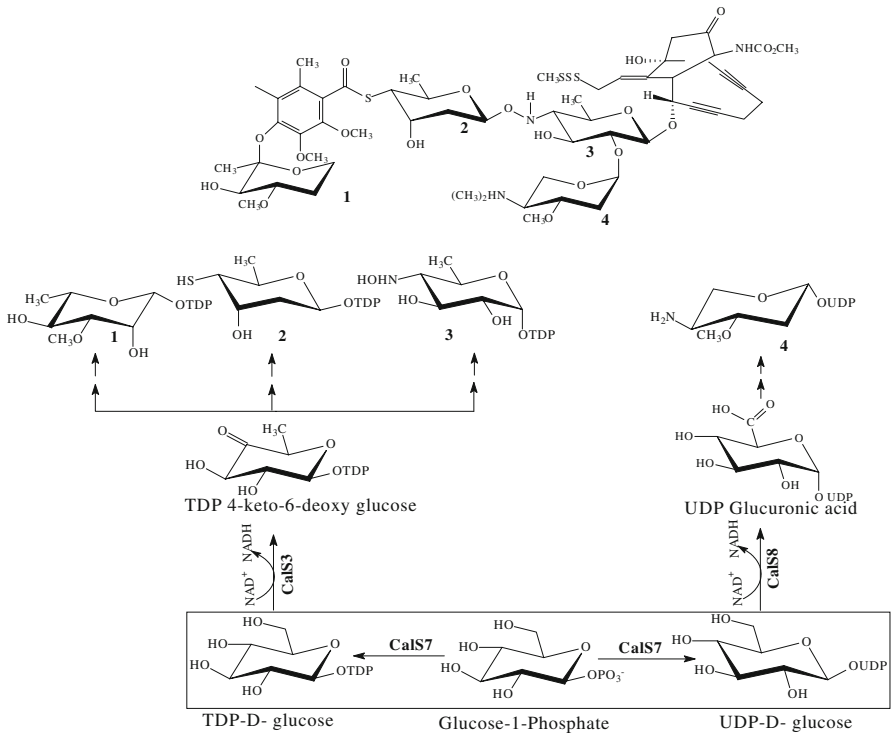

Fig. 1 Structure of calicheamicin and the proposed biosynthetic pathway of the deoxysugars. **1**, 3-methoxy-L-rhamnose; **2**, TDP-4-hydroxylamine-2,4,6-trideoxy-D-glucose; **3**, TDP-4-thio-2,4,6-trideoxy-D-glucose; and **4**, UDP-4-amino-3-*O*-methoxy-2,4,5-trideoxypentose

synthase). The NDP-glucose synthase is found within gene clusters that encode the biosynthetic enzymes for natural products containing deoxyhexose moieties (Pissowotzki et al. 1991; Merson-Davies and Cundliffe 1994; Marolda and Valvano 1995; Sohng et al. 2002).

The calicheamicin gene cluster contains a single nucleotidyltransferase gene (*calS7*) for the synthesis of both TDP-sugars (3-methoxy-L-rhamnose; TDP-4-hydroxylamine-2,4,6-trideoxy-D-glucose; TDP-4-thio-2,4,6-trideoxy-D-glucose) and the UDP-sugar (UDP-4-amino-3-*O*-methoxy-2,4,5-trideoxypentose) (Ahlert et al. 2002). Therefore, the *calS7* gene present in the calicheamicin gene cluster is proposed to be involved in the transfer of a nucleotidyl group in glucose-1-phosphate during the biosynthesis of sugar moieties (Fig. 1).

Since CalS7 showed putative thymidylyltransfrase based on BLAST analysis from NCBI database, we individually annotated CalS7 with several proteins which were already characterized as uridyltransferases (Aragäo et al. 2006; Thoden and Holden 2007a, b) in order to know the possible putative products. From sequence analysis it is revealed that CalS7 also showed significance similarity with uridyltransferase from several strains like *Corynebacterium glutamicum* ATCC 13032 (GenBank accession no. NP_599583), *Yersinia pestis* CO92 (GenBank accession no. NP_405125) and *E. coli* K12 (GenBank accession no. NP_415752). Sequence analysis of CalS7 provided us strong bootstrap support for heterologous expression. In order to verify the function of it, we cloned and heterologously expressed in *E. coli* BL21(DE3) and in vitro enzyme assay was carried out. Our results showed that CalS7 is a key enzyme for the biosynthesis of TDP and UDP-sugar moieties present in calicheamicin.

Materials and methods

Bacterial strains, growth conditions, and plasmids

Escherichia coli XL1-Blue MRF′ was used as a host cell for the preparation of recombinant plasmids, and *E. coli* BL21(DE3) (Stratagene, USA) was used for the expression of the enzyme. Synthetic oligonucleotides were obtained from the Bioneer Co. (Daejeon, Korea). All chemicals used in this study were purchased from Sigma (St. Louis, USA). The disodium salt of glucose-1-phosphate and the disodium salt of ATP, TMP, UMP, TTP, UTP, CTP, GTP and

$MgCl_2 \cdot 6H_2O$ were used for the enzyme reaction. The pGEM-T Easy (Promega, USA) and pET32a(+) (Novagen, Germany) vectors were used as vectors for cloning and expression, respectively. For the selection and maintenance of plasmids, *E. coli* was grown at 37°C in Luria-Bertani (LB) broth or on an agar plate supplemented with the appropriate amount of antibiotics when necessary (ampicillin up to 50 μg ml^{-1}). The pET15b plasmid containing the pBR322 origin of replication and the ampicillin resistance gene was used to clone the TMP kinase, and the pET24ma vector containing the p15A origin and kanamycin resistance gene was used to clone acetate kinase (E.C.2.7.2.1). TMP kinase (E.C.2.7.4.9) and acetate kinase were amplified by polymerase chain reaction (PCR) using the genomic DNA of *E. coli* K12 as a template. All of the genes were cloned under the control of the T7 promoter (Oh et al. 2003).

DNA manipulation and construction of pCalS7

DNA manipulations, restriction endonuclease digestion, and ligation were carried out according to standard protocols. Lysozyme treatment of *M. echinospora* and phenol-chloroform extraction were performed as described by Kieser et al. (2000). The recombinants were constructed for *E. coli*. Two synthesized oligonucleotide primers, CalS7F (5′-GCT GAA TTC ATG CGT GGT GTT TTG CT-3′) and CalSR (5′-ATT AAG CTT TCA CCC GAC GGC GGC C- 3′), were used to amplify *calS7*. The PCR product (894 bp) was cloned into the *Eco*RI and *Hin*dIII sites of pET32a(+) in order to generate pCalS7. To verify that no mutation had been introduced during the PCR amplification, the PCR products were cloned into the pGEM-T Easy vector and sequenced before cloning into the expression vector. PCR was performed using a thermocylcer (Takara, Japan) under the following conditions: 30 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C. The recombinant expression vector was introduced into *E. coli* BL21 (DE3) by heat-pulse transformation, and the antibiotic resistant transformants were selected.

Expression and purification of CalS7

Escherichia coli BL21 (DE3) harboring the recombinant plasmid pCalS7 was grown in 3 ml LB culture medium. The overnight culture medium was transferred to 50 ml fresh LB medium containing ampicillin and then grown at 37°C and 250 rpm. At an OD_{600} of 0.6, IPTG was added to give 0.4 mM, and the incubation was continued for 20 h at 20°C. Thawed cell pellets were harvested by centrifugation at 6,000 × *g* for 10 min. They were also washed twice with 20 ml of 50 mM Tris/HCl (pH 7.5) and sonication buffer containing 1 mM PMSF, 1 mM DTT, 100 mM EDTA (pH 8), 1 M $MgCl_2$, 50 mM Tris/HCl (pH 7.5) and 10% glycerol. The cells were lysed by sonication on ice with an ultra-sonicator. The debris was removed by centrifugation at 12,000 × *g* for 40 min at 4°C.

The His-tagged CalS7 fusion soluble protein was purified by Co^{2+} affinity chromatography (TALON Purification Kit, Clontech, USA) according to the instructions supplied by the manufacturer. The purified fractions were desalted by dialysis and concentrated using Centricon (Ultracel YM-10, Millipore). The molecular weight of the proteins was analyzed and determined by 12% (v/v) SDS-PAGE using standard molecular weight protein markers. The separating and stacking gels consisted of 12 and 5% polyacrylamide, respectively. Protein concentration was determined according to the Bradford method using the Bio-rad protein assay with bovine serum albumin (BSA) as the standard, and a protein concentration of 65 μg/ml was obtained.

Enzyme assay and analysis of CalS7

A typical enzyme assay condition for CalS7 involves 100 μl containing 50 mM Tris/HCl (pH 7.5), 5 mM NTP, 5 mM glucose 1-phosphate, 1.8 U inorganic pyrophosphase, and 25 mM $MgCl_2$. The reaction was carried out for 30 min at 37°C. The reaction was quenched by heating the sample at 90°C for 45 s, followed by centrifugation for 10 min at 4°C. The supernatant was then obtained for HPLC analysis. The concentrations of UMP/TMP, acetyl phosphate, glucose 1-phosphate, ATP and $MgCl_2$ used for the one-pot enzymatic assay of CalS7 were 5, 50, 45, 1 and 20 mM, respectively. The reaction was initiated by using 0.5 U TMP kinase, 50 U acetate kinase and glucose-1-phosphate nucleotidylyltransferase (CalS7) at 37°C for 90 min (Oh et al. 2003). The reaction was stopped by heating the reaction mixture at 90°C for 45 s, and the precipitate was removed by centrifugation at

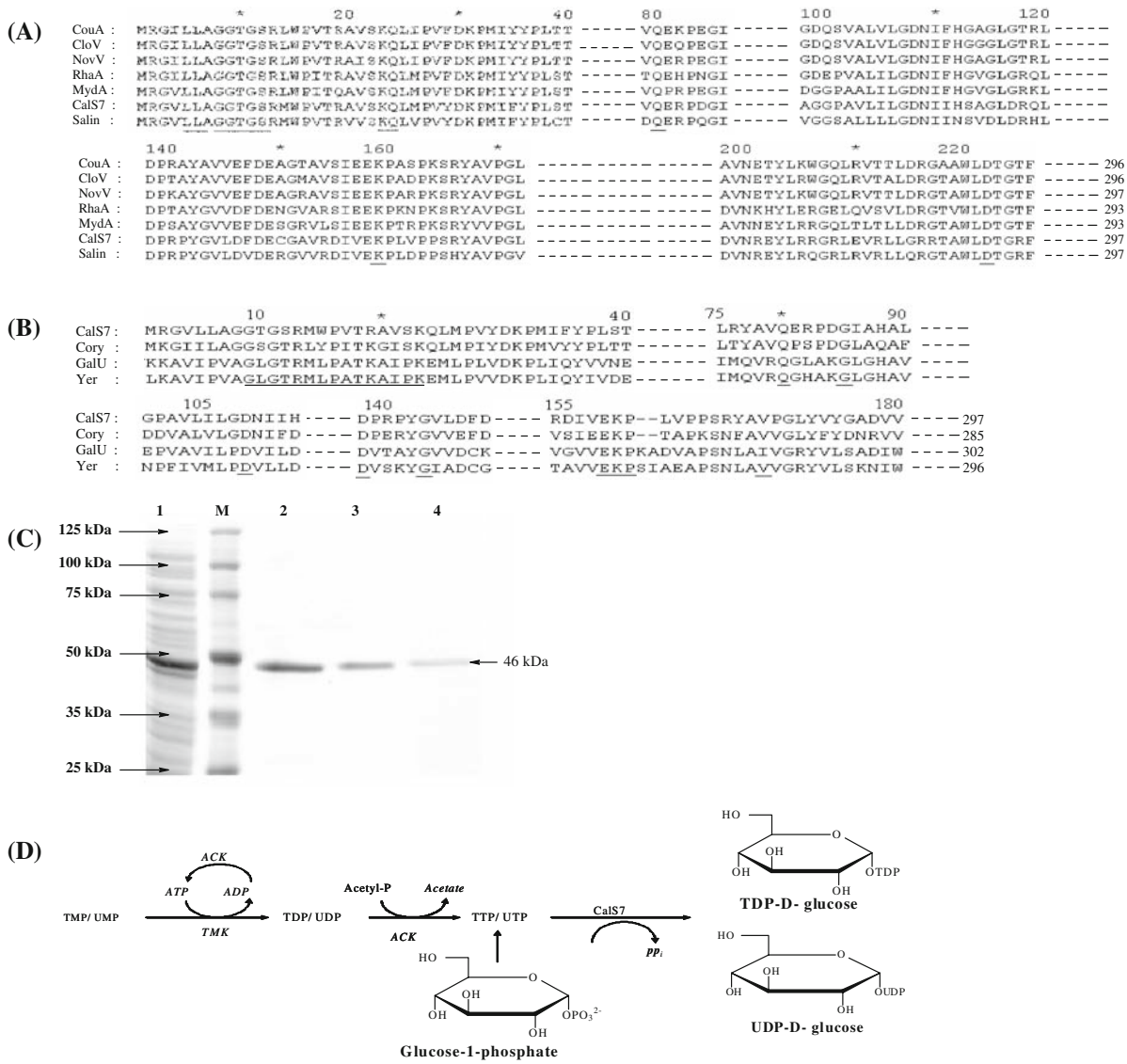

Fig. 2 (**a**) Multi-alignment analysis of deduced amino acid sequence of CalS7 with its thymidylyltransferase homology: 81% identity with *Salinispora arenicola* CNS-205 (GenBank accession no. YP_001537037), 69% identity with MydA of *Micromonospora griseorubida* (GenBank accession no. BAC57039), 68% identity with RhaA of *S. olivaceous* (GenBank accession no. CAP11385), 61% identity with CouV of *S. rishiriensis* (GenBank accession no. AAG29804) 61% identity with CloV of *S. roseochromogenes* subsp. *oscitans* (GenBank accession no. AAN65244) and 60% identity with NovV of *S. caeruleus* (GenBank accession no. AAF67515), (**b**) Multiple sequence alignment of CalS7 with its uridylyltransferase homology: 48% identity with *Corynebactericum glutamicum* ATCC 13032 (GenBank accession no. NP_599583), 36% with *Yersinia pestis* CO92 (GenBank accession no. NP_405125) and 33% with *E. coli* K12 (GenBank accession no. NP_415752). Catalytic motifs and conserved regions are underlines and numbers are given from *M. echinospora* amino acid sequence, (**c**) SDS-PAGE of CalS7. Lane 1, soluble protein; M, Marker; 2, 3 and 4, after elution with 100 mM immidazole buffer, (**d**) One-pot reaction mechanism using TMP, acetyl phosphate, TMK, ACK and CalS7 for the synthesis of TDP-glucose, and UMP, acetyl phosphate, TMK, ACK and CalS7 for the synthesis of UDP-glucose

12,000 × *g* for 10 min. The supernatant was analyzed by electrospray ionization-mass spectroscopy (ESI/MS) (Thermo Finnigan, USA) and HPLC. HPLC analysis was carried out at 254 nm using a C18 column (C18 XTerra RP-18, 5 μm 5 × 250 mm, Waters). Isocratic elution was with 100 mM potassium

phosphate buffer (pH 7.0) containing 8 mM tetra-*n*-butyl ammonium hydrogen sulfate and methanol (95:5 v/v) at 1 ml min^{-1}.

Table 1 Conversion ratio of several nucleotides to their respective sugar

Nucleotides (5 mM)	Relative activity with glucose 1-phosphate (5 mM)
TTP	1 ± 0.02
UTP	1.08 ± 0.03
ATP	0.008 ± 0.00
CTP	0.001 ± 0.00
GTP	0.32 ± 0.01

Results and discussion

CalS7 shows a high degree of amino acid sequence similarity and conserved motifs with other thymidylyltransferase genes in the data base according to BLAST searches (Fig. 2a). Similarly, it also shows significant homology to uridylyltransferase from several strains (Fig. 2b). Based on homology study and conserved motifs, CalS7 should function thymidylytransferase and uridylyltransferase.

The PCR product (894 bp) of *calS7* was cloned into the *Eco*RI/*Hin*dIII sites of pET32a(+) to generate pCalS7. The recombinant pCalS7 was transferred and expressed in a heterologous host strain, *E. coli*

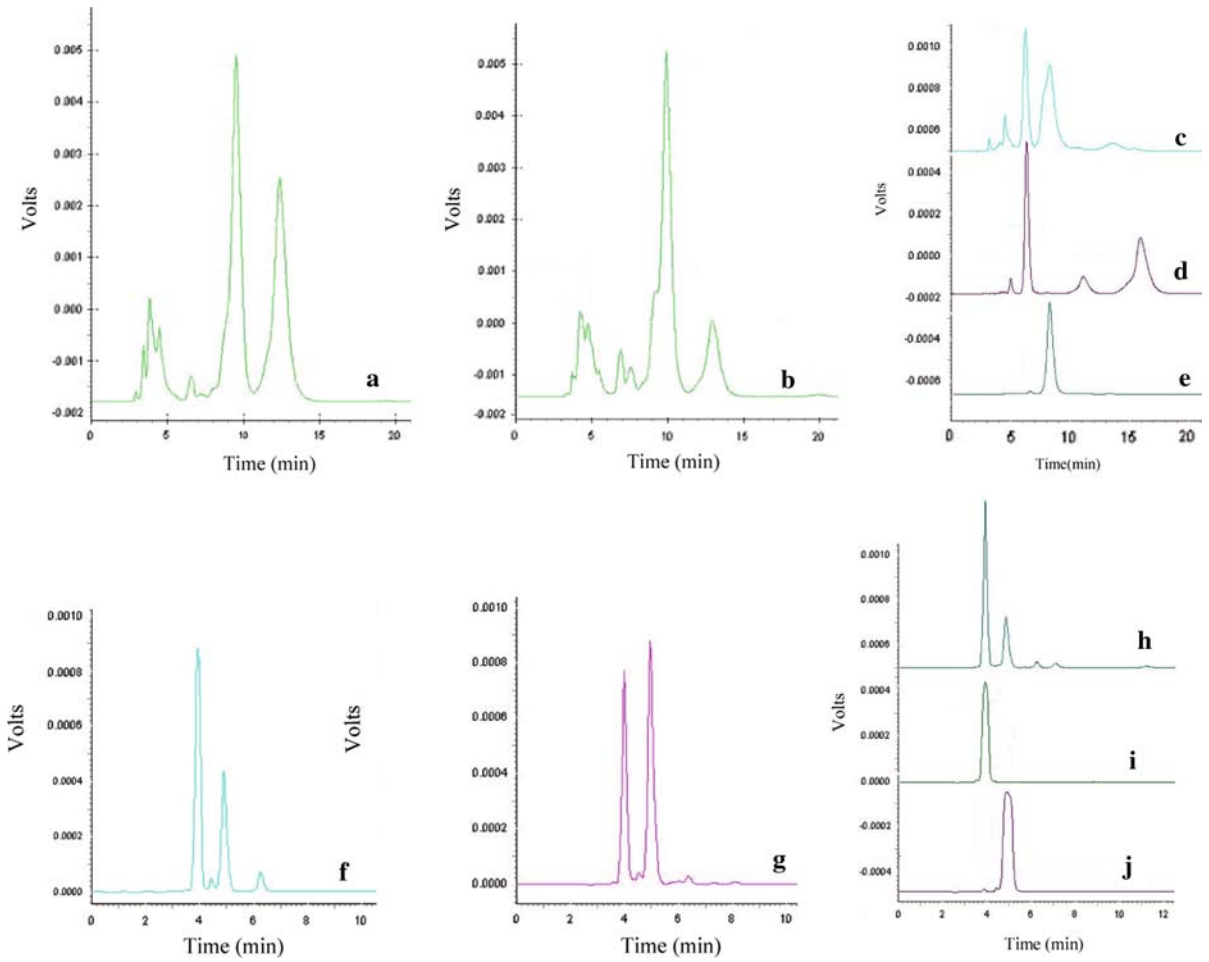

Fig. 3 HPLC analysis. (**a**, **b**) Profile of one-pot enzymatic assay with CalS7, TTP and glucose-1-phosphate after 30 and 90 min, respectively. (**c**) Profile after one-pot enzymatic synthesis in TDP-glucose. (**d**) Profile of control where no product seems to be appeared. (**e**) TDP-glucose standard. (**f**, **g**) Profile of one-pot enzymatic assay with CalS7, UTP and glucose-1-phosphate after 30 and 90 min, respectively. (**h**) Profile after one-pot enzymatic synthesis in UDP-glucose. (**i**, **j**) Comparison of product with standard sample with UMP and UDP-glucose, respectively

BL21(DE3). Expression with His-fusion protein in soluble form was achieved at 20°C after induction with 0.4 mM IPTG for 20 h. During the purification process, the target proteins were completely eluted at a concentration of 100 mM imidazole in potassium phosphate buffer (pH 6.0), and the molecular weights of the denatured proteins determined by SDS-PAGE analysis (Fig. 2c) were in good agreement with those of the calculated values of ~46 kDa.

The reaction used to demonstrate the predicted function of CalS7, which catalyzes the conversion of nucleotides and glucose-1-phosphate to their respective glucose residues, was performed as described in the experimental section. The conversion ratio of TTP, UTP, CTP, GTP and ATP to their respective glucose residues in the presence of CalS7 was calculated, as was that of glucose-1-phosphate (Table 1). The conversion ratio of UTP and glucose 1-phosphate to UDP-glucose appears to be higher than that of TDP-glucose; however, GTP-glucose was observed in a significantly greater amount than the CTP and ATP nucleotides. These results indicate that CalS7 is the precursor responsible for the biosynthesis of deoxyhexose and deoxypentose in calicheamicin.

In addition, a one-pot enzymatic assay was also carried out using TMK, ACK and CalS7, starting with UMP and TMP to obtain UDP-glucose and TDP-glucose, respectively. TMP is the starter substrate for TDP-glucose, whereas UMP is the starter substrate for UDP-glucose. In the presence of thymidylyl kinase, TMP/UMP is converted into TDP/UDP, which provides further direction for the formation TTP/UTP and leads to the production of the final products in the presence of CalS7 (Fig. 2d). Acetate kinase was used in excess to ensure the efficient regeneration of ATP and to prevent the accumulation of UDP and TDP. The enzyme assay was carried out for various time periods in order to determine the conversion rate. The reaction was stopped by heating at 90°C for 45 s and the supernatant was analyzed by HPLC.

The HPLC profiles shown in Fig. 3a, b represent the conversion of TTP to TDP-glucose in the presence of glucose 1-phosphate at 30 and 90 min, respectively, and the HPLC profiles shown in Fig. 3c–e represent the one-pot enzymatic synthesis after 90 min, control profile, and standard TDP-glucose, respectively. Similarly, the HPLC profile was also analyzed by one-pot enzymatic synthesis of UDP-glucose. Figure 3f, g represent the HPLC profile after 30 and 90 min, respectively, and Fig. 3h–j are the profiles in comparison to the respective substrate (3i, UMP-glucose; 3j, UDP-glucose).

These profiles show the clear formation of TDP- and UDP-glucose after 90 min of incubation. ESI/MS analysis of the resulting products showed mass peaks of $m/z = 563$ and 565 for TDP-glucose and UDP-glucose, respectively (Fig. 4).

The calicheamicin gene cluster contains a single nucleotidyltransferase gene (CalS7) used for the synthesis of both TDP- and UDP-sugars. Gene analysis revealed that the conserved domains that are responsible for thymidylyltransferase and uridylyltransferase are present. Nucleotide reactions and one-pot synthesis were also carried out for the characterization of CalS7 by HPLC and ESI/MS analysis. The results showed that CalS7 is the nucleotidyltransferase gene involved in the biosynthesis of deoxyhexose and deoxypentose sugars in calicheamicin. This research provides the precursor for the biosynthesis of 3-methoxy-L-rhamnose

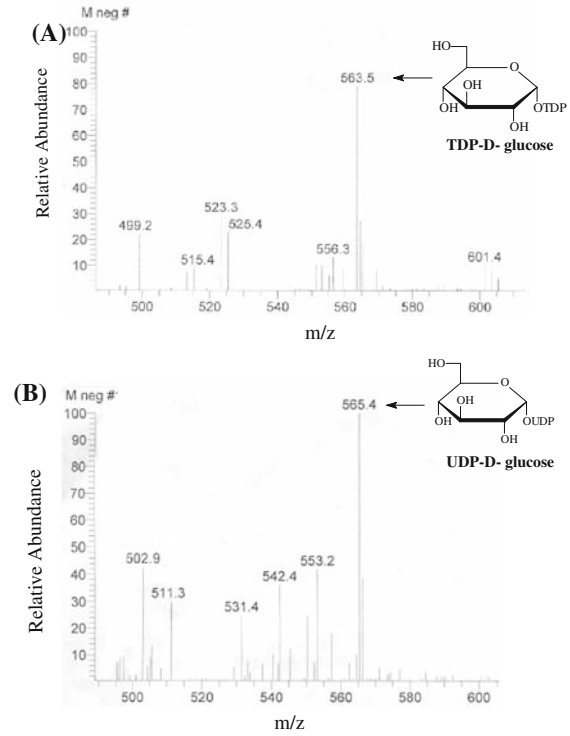

Fig. 4 ESI/MS analysis in negative mode $m/z = 563$, TDP-D-glucose product (**a**), and $m/z = 565$, UDP-D-glucose product (**b**)

containing TDP-4-hydroxylamine-2,4,6-trideoxy-D-glucose, TDP-4-thio-2,4,6-trideoxy-D-glucose, and UDP-4-amino-3-*O*-methoxy-2,4,5-trideoxy pentose, and it also advances the production of other activated nucleotide-linked sugars, which are absolutely required for the biosynthesis of the unusual deoxysugars in certain antibiotics.

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References

Ahlert J, Shepard E, Lomovskaya N et al (2002) The calicheamicin gene cluster and its iterative type I enediyne PKS. Science 297:1173–1176

Aragäo D, Marques AR, Frazäo C et al (2006) Cloning, expression, purification, crystallization and preliminary structure determination of glucose-1-phosphate uridylyltransferase (UgpG) from *Sphingomonas elodea* ATCC 31461 bound to glucose-1-phosphate. Acta Crystallogr Sect F Struct Biol Cryst Commun 62:930–934

Barton WA, Lesniak J, Biggins JB et al (2001) Structure, mechanism and engineering of a nucleotidylyltransferase as a first step toward glycorandomization. Nat Struct Biol 8:545–551

Biggins JB, Onwuemw KC, Thorson JS (2003) Resistance to enediyne antitumor antibiotics by CalC self-sacrifice. Science 301:1537–1541

Blankenfeldt W, Asuncion M, Lam JS et al (2000) The structural basis of the catalytic mechanism and regulation of glucose-1-phosphate thymidylyltransferase (RmlA). EMBO J 19:6652–6663

Galm U, Hager MH, Van Lanen SG et al (2005) Antitumor antibiotics: bleomycin, enediynes, and mitomycin. Chem Rev 105:739–758

Kieser T, Bibb MJ, Buttner MJ et al (2000) Practical streptomyces genetics. John Innes Foundation, Norwich

Marolda CL, Valvano MA (1995) Genetic analysis of the dTDP-rhamnose biosynthesis of the *E. coli* Vw 187 (O7:K1) *rfb* gene cluster: identification of functional homologs of *rfbB* and *rfbA* in the *rff* cluster and correct location of the *rffE* gene. J Bacteriol 177:5539–5546

Merson-Davies LA, Cundliffe E (1994) Analysis of five tylosin biosynthesis genes from the *tylBA* region of the *Streptomyces fradie* genome. Mol Microbiol 13:349–355

Myers AG, Cohen SB, Kwon BM (1994) DNA Cleavage by neocarzinostatin chromophore establishing the intermediacy of chromophore-derived cumulene and biradical species and their role in sequence-specific cleavage. J Am Chem Soc 116:1255–1271

Oh J, Kim BG, Sohng JK et al (2003) One-pot enzymatic production of dTDP-4-keto-6-D-glucose from dTMP and glucose-1-phosphate. Biotech Bioeng 84:452–458

Parajuli N, Lee DS, Lee HC et al (2004) Cloning, expression and characterization of glucose-1-phosphate thymidylyltransferase (*strmlA*) from *Thermus caldophilus*. Biotechnol Lett 26:437–442

Pissowotzki K, Mansouri K, Piepersberg W (1991) Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative function of genes *strELMB2N*. Mol Gen Genet 231:113–123

Simone Z, Davide Z, Camillo R et al (2001) Kinetic and crystallographic analyses support a sequential-ordered Bi catalytic mechanism for *Escherichia coli* glucose-1-phosphate thymidylyltransferase. J Mol Biol 313:831–843

Sohng JK, Noh HR, Lee OH et al (2002) Function of lysine-148 in TDP-D-glucose 4, 6-dehydratase from Streptomyces antibioticus Tű99. J Microbiol Biotechnol 12:217–221

Thoden JB, Holden HM (2007a) Active site geometry of glucose-1-phosphate uridylyltransferase. Protein Sci 16:1379–1388

Thoden JB, Holden HM (2007b) The molecular architecture of glucose-1-phosphate uridylyltransferase. Protein Sci 16:432–440