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Carbohydrate Research 335 (2001) 23–32

CARBOHYDRATE RESEARCH

(Chemo)enzymatic synthesis of dTDP-activated 2,6-dideoxysugars as building blocks of polyketide antibiotics

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Received 11 April 2001; accepted 10 July 2001

Abstract

The flexible substrate spectrum of the recombinant enzymes from the biosynthetic pathway of dTDP-β-L-rhamnose in *Salmonella enterica*, serovar typhimurium (LT2), was exploited for the chemoenzymatic synthesis of deoxythymidine diphosphate- (dTDP-) activated 2,6-dideoxyhexoses. The enzymatic synthesis strategy yielded dTDP-2-deoxy-α-D-glucose and dTDP-2,6-dideoxy-4-keto-α-D-glucose (**13**) in a 40–60 mg scale. The nucleotide deoxysugar **13** was further used for the enzymatic synthesis of dTDP-2,6-dideoxy-β-L-*arabino*-hexose (dTDP-β-L-olivose) (**15**) in a 30-mg scale. The chemical reduction of **13** gave dTDP-2,6-dideoxy-α-D-*arabino*-hexose (dTDP-α-D-olivose) (**1**) as the main isomer after product isolation in a 10-mg scale. With **13** as an important key intermediate, the in vitro characterization of enzymes involved in the biosynthesis of dTDP-activated 2,6-dideoxy-, 2,3,6-trideoxy-D- and L-hexoses can now be addressed. Most importantly, compounds **1** and **15** are donor substrates for the in vitro characterization of glycosyltransferases involved in the biosynthesis of polyketides and other antibiotic/antitumor drugs. Their synthetic access may contribute to the evaluation of the glycosylation potential of bacterial glycosyltransferases to generate hybrid antibiotics.

Keywords: Nucleotide deoxysugars; dTDP-β-L-olivose; dTDP-α-D-olivose; Antibiotics; Macrolides

1. Introduction

D- and L-deoxyhexoses are important constituents of glycoconjugates with biological functions in animals, plants, and microorganisms.[1] In general, all of them can be classified as 6-deoxy-D- and L-hexoses. Deoxygenation at C-2, C-3, and C-4 of the 6-deoxy-hexoses and modifications by amino-, *N*-methyl-, *O*-methyl-, and *C*-methyl-groups create a structural diversity, which is encoded by distinct biosynthetic pathways of deoxythymidine diphosphate- (dTDP-), cytidine diphosphate- (CDP-), and guanosine diphosphate- (GDP-) activated sugars and the substrate specificity of glycosyltransferases.[2,3] In secondary metabolites of actinomycetes D- and L-deoxyhexoses contribute to their antibiotic and antitumor bioactivity. Among the polyketide antibiotics these are mainly 2,6-dideoxyhexoses besides 2,3,6-trideoxy sugars, which are dTDP-activated and donor substrates of

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0008-6215/01/$ - see front matter
PII: S0008-6215(01)00195-1

glycosyltransferases (Table 1 and Scheme 1). The biosynthetic pathways of these D- and L-deoxyhexoses start with the conversion of α-D-glucose-1-phosphate (**9**) and dTTP to yield the precursor dTDP-α-D-glucose (dTDP-Glc) (**10**) and the common intermediate dTDP-4-keto-6-deoxyglucose (**11**) (Scheme 2).[4–7]

Knowledge about the biosynthetic pathways beyond the central intermediate has been further expanded by analyses of the gene clusters of the antibiotic strains.[8] However, the order of enzymatic conversions in the biosynthetic routes is only recently starting to be elucidated by in vitro studies with recombinant enzymes. It has been clearly demonstrated that the 2,6-dideoxyhexoses biosynthesis proceeds from **11** with the formation of dTDP-3,4-diketo-2,6-dideoxyglucose (**12**) (Scheme 2). The stereospecific reduction of the unstable intermediate **12** by 3-ketoreductases yields dTDP-2,6-dideoxy-4-ketoglucose (**13**), which is the central intermediate of the dTDP-2,6-dideoxyhexoses **1**, **2**, **6**, **8** and dTDP-L-olivose (**15**).[9–11]

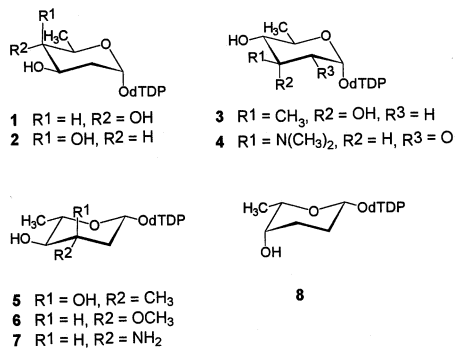
Similar biochemical functions were attributed to the enzymes involved in the biosynthesis of dTDP-L-mycarose (**5**) in the tylosin biosynthetic cluster.[12] However, the corresponding 3-ketoreductase forms an axial instead of an equatorial hydroxyl group at C-3 resulting in dTDP-2,6-dideoxy-4-keto-D-allose (**16**) as intermediate (Scheme 2). Although the same biochemical functions can now be assigned to the enzymes in the erythromycin biosynthetic cluster,[13–16] the exact order and stereochemistry of the products beyond intermediate **16** have still to be determined. In summary, more in vitro biochemical studies are needed for an indepth understanding of the biosynthetic routes and for the engineering of these metabolic pathways. This goal is still hampered by the limited access to important dTDP-deoxyhexoses in a preparative synthesis scale.

In this context, the aim of our work is to provide precursors, central intermediates of dTDP-deoxyhexose pathways, as well as donor substrate of glycosyltransferases for biochemical studies.[17] In contrast to the described pathways, we followed alternate synthetic routes employing sucrose synthase (EC 2.4.1.13; SuSy) in order to establish a novel access to nucleotide sugars from sucrose and nucleoside diphosphates (NDP).[18] dTDP-Glc (**10**) was the first nucleotide sugar which was synthesized in a continuous mode in an enzyme membrane reactor.[19] The combination of SuSy with the recombinant dTDP-D-glucose-4,6-dehydratase (RmlB, EC 4.2.1.46) from the dTDP-L-rhamnose (dTDP-Rha) pathway yielded **11**.[20] The enzymatic reactions were optimized to a g-scale synthesis of this central intermediate.[21]

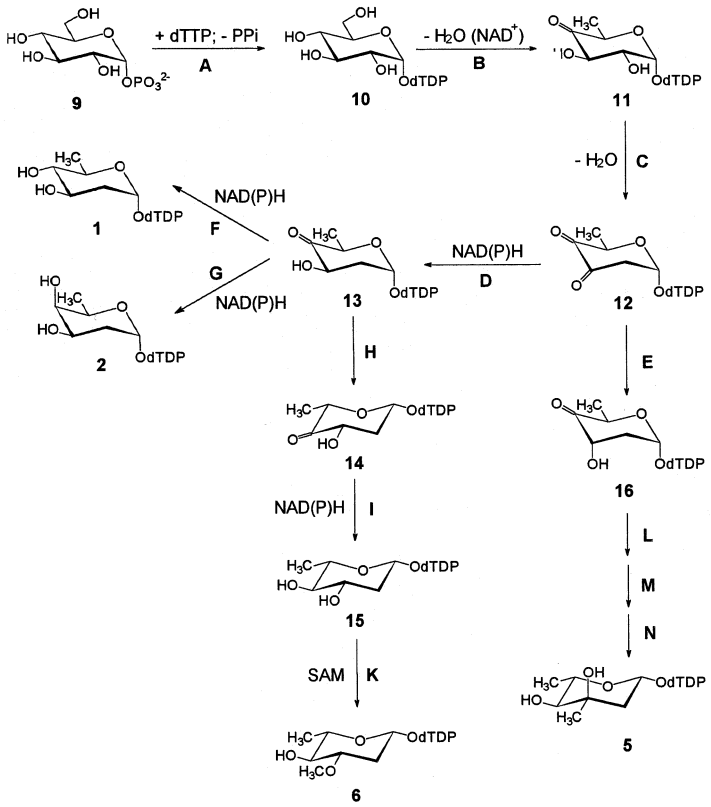
In the present paper we have exploited the flexible substrate spectrum of phosphoglucomutase and the enzymes from the dTDP-Rha pathway.[7,22,23] Although the C-2 hydroxyl group was reported to be critical for conversion by RmlA (dTDP-glucose pyrophosphorylase, EC 2.7.7.24),[24] dTDP-2-deoxy-glucose (**17**) was synthesized by the conversion of 2-deoxy-glucose-6-phosphate (**18**) with phos-

Table 1
dTDP-activated deoxyhexoses as donor substrates of glycosyltransferases in the biosynthetic pathways of polyketide antibiotics

dTDP-deoxyhexose	Polyketide antibiotic
dTDP-D-olivose (**1**)	mithramycin, landomycin A, urdamycin A
dTDP-D-oliose (**2**)	mithramycin
dTDP-D-mycarose (**3**)	mithramycin
dTDP-D-mycaminose (**4**)	tylosin, midecamycin
dTDP-L-mycarose (**5**)	tylosin, midecamycin, erythromycin D
dTDP-L-oleandrose (**6**)	oleandomycin, avermectin B_{1a}
dTDP-L-daunosamine (**7**)	daunomycin
dTDP-L-rhodinose (**8**)	landomycin A, urdamycin A



Scheme 1. dTDP-activated deoxyhexoses occurring in deoxysugar pathways of polyketide antibiotics.



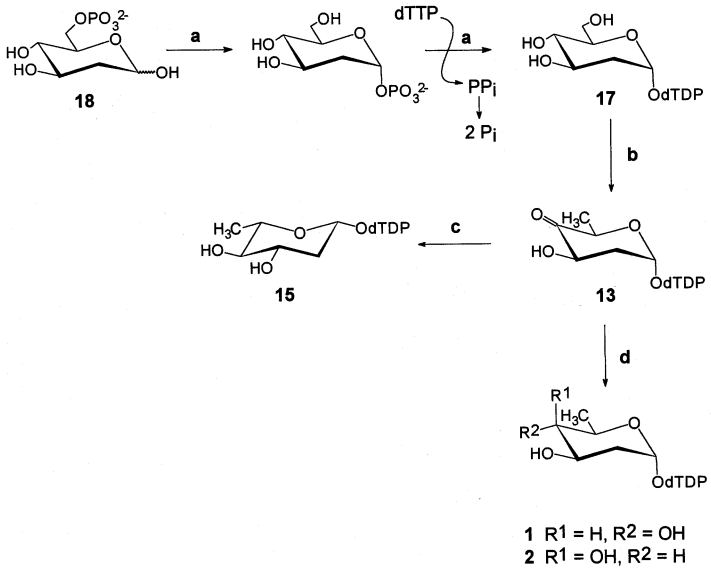
Scheme 2. Proposed biosynthetic pathways of dTDP-2,6-dideoxy-D- and L-hexoses. The indicated enzymes are involved in deoxysugar pathways from *Streptomyces* strains producing different polyketide antibiotics. (A) dTDP-Glc pyrophosphorylase (EC 2.7.7.24): OleS, Kde, DesIII, TylA1, AveBIII, LanG, MtmD. (B) dTDP-Glc 4,6-dehydratase (EC 4.2.1.46): OleE, Gdh, TylA2, DesIV, AveBII, MtmE. (C) dTDP-4-keto-6-deoxyGlc 2,3-dehydratase: OleV, EryBVI, GraOrf27, Tü99Orf10, TylX3, AveBVI, MtmV. (D) dTDP-3,4-diketo-2,6-dideoxyGlc 3-reductase: OleW, GraOrf26, Tü99Orf11, AveBVIII, MtmW. (E) dTDP-3,4-diketo-2,6-dideoxyGlc 3-reductase: EryBII, TylC1. (F) 4-ketoreductase: LanT, AviJ. (G) 4-ketoreductase: MtmU. (H) 3,5-Epimerase: OleL, AveBV. (I) 4-Ketoreductase: OleU, AveBIV. (K) *O*-Methyltransferase: OleY, AveBVII. (L) 3,5-Epimerase: EryBVII. (M) *C*-Methyltransferase: EryBIII. (N) 4-Ketoreductase: EryBIV.

phoglucomutase (PGM, EC 5.4.2.2) and RmlA (Scheme 3). Subsequent conversion of **17** with RmlB (dTDP-Glc-4,6-dehydratase, EC 4.2.1.46) yielded dTDP-2,6-dideoxy-4-ketoglucose (**13**). dTDP-L-olivose (**15**) was obtained by reaction with RmlC (dTDP-4-dehydrorhamnose 3,5-epimerase, EC 5.1.3.13) and RmlD (dTDP-4-dehydrorhamnose reductase, EC 1.1.1.133). A chemical reduction of **13** with $NaBH_4$ yielded dTDP-D-olivose (**1**) as the major product.

2. Results and discussion

The enzymatic synthesis of dTDP-2,6-dideoxyhexoses was accomplished by a three-step synthesis strategy as depicted in Scheme 3.

Enzymatic synthesis and isolation of dTDP-2-deoxy-Glc.—The first step was the synthesis of **17**. The unfavorable equilibrium of the PGM catalyzed reaction afforded 12 mM of the substrate **18** and 0.6 U mL^{-1} enzyme for an optimal reaction in combination with RmlA and pyrophosphatase (PPase). The activity of RmlA was strongly dependent on the ratio of Mg^{2+}/dTTP. Fig. 1 illustrates that the optimal activity for RmlA was at a Mg^{2+}/dTTP ratio of 2.5. This result confirms the common feature of pyrophosphorylases to be dependent on the Mg^{2+}/NTP ratio as demonstrated recently for UDP-Glc pyrophosphorylase from malt[25] and GDP-Man pyrophosphorylase (RfbM) from *Salmonella typhimurium* LT2.[26] The combination of RmlA with RmlB was avoided. Preliminary experiments demonstrated that RmlA was inhibited by dTDP-4-keto-6-deoxy-Glc, the product of RmlB, with an IC_{50} value of 1.5 mM. RmlB was inhibited by dTTP, the sub-



Scheme 3. Strategy for the chemoenzymatic synthesis of dTDP-2,6-dideoxyhexoses. (a) 50 mL batch with 0.6 U mL^{-1} PGM, 3 μM Glc-1,6-P_2, 12 mM **18**, 2 mM dTTP, 5 mM $MgCl_2$, 3 U mL^{-1} RmlA, 10 U mL^{-1} PPase in 50 mM Tris–HCl pH 8.0 at 30 °C for 3 h. Ultrafiltration and repetitive use of the enzymes by addition of fresh substrate solution. (b) 50 mL batch with 3.36 mM **17**, 10 U mL^{-1} RmlB, 2 U mL^{-1} alkaline phosphatase in 50 mM Tris–HCl pH 8.0 at 30 °C for 2 h. Ultrafiltration and repetitive use of the enzymes by addition of fresh substrate solution. (c) 44 mL batch with 2.0 m **13**, 2.0 mM NADPH, 0.55 U mL^{-1} RmlC, 4.3 U mL^{-1} RmlD in 50 mM Tris–HCl pH 8.0 at 30 °C for 5 h. Addition of 2 U mL^{-1} alkaline phosphatase and incubation for 2 h, ultrafiltration, and product isolation. (d) 2 mL batch with 17.75 mM **13**, in distilled water, addition of three aliquots of $NaBH_4$ (53.5 μmol) at pH 7.5 during 5 h. Addition of 2 U mL^{-1} alkaline phosphatase and incubation for 2 h, ultrafiltration, and product isolation.

strate of RmlA, with an IC_{50} value of 0.5 mM. In addition dTDP, a possible decomposition product of **13**, is a strong inhibitor of RmlB (K_i 2.5 μM).[21] These results led to an optimized preparative synthesis of **17**, which was performed in a repetitive-batch mode. Our previous work demonstrated that the repetitive use of enzymes increases the enzymes' specific productivities.[21,27–31] In five batches with an incubation time of 16 h, **17** was synthesized with an average yield of 86%, as analyzed by HPLC (with reference to dTTP). Product isolation by ultrafiltration for removal of proteins, anion-exchange chromatography and gel filtration gave 60.8 μmol (36 mg, 61% overall yield) of **17** (sodium salt) with a purity of 94% according to HPLC. The product was characterized as dTDP-2-deoxy-α-D-*arabino*-hexose by 1D/2D NMR spectroscopy. The integrity of the nucleoside diphosphate-α-D-deoxysugar was indicated by the typical heterocoupling constants $^3J_{H\text{-}1,P}$ 6.8 and $^4J_{H\text{-}2ax,P}$ 3.0 Hz in the ^1H NMR spectrum and $^2J_{C\text{-}1,P}$ 6.0 and $^3J_{C\text{-}2,P}$ 7.5 Hz in the ^{13}C NMR spectrum.[19] The α configuration of the hexose was additionally proven by the vicinal coupling constants of the trans equatorial protons with $^3J_{1,2eq}$ 0.8 Hz, and the cis axial–equatorial protons with $^3J_{1,2ax}$ 3.0 Hz. The typical high coupling constants for both protons at C-2 with $^2J_{2eq,2ax}$ 13.2 Hz and the coupling of the equatorial H-2 with the neighbored H-1 and H-3 protons, $^3J_{1,2eq}$ 0.8 and $^3J_{2eq,3}$ 4.6 Hz, are due to the 2-deoxygenation of the hexose moiety. The ^{31}P-(^1H-coupled) NMR-spectrum gave the characteristic chemi-

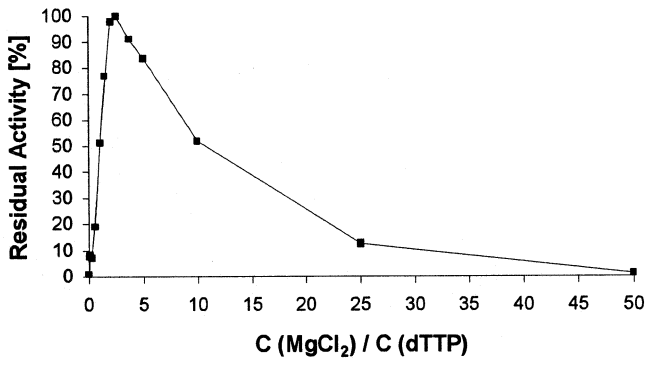


Fig. 1. Effect of the Mg^{2+}/dTTP ratio on the activity of RmlA.

cal shifts with the typical coupling constants of $^{2}J_{P,P}$ 20.3 Hz for the glycosyl phosphate dTDP-2-deoxy-α-D-glucose.

Enzymatic synthesis and isolation of dTDP-2,6-dideoxy-4-ketoglucose.—In a second synthesis, 168 μmol of **17** was synthesized as described above. The synthesis of **13** was performed in a repetitive batch mode after removal of enzymes by ultrafiltration and addition of RmlB and alkaline phosphatase (Scheme 3). The latter enzyme was necessary to hydrolyze dTDP, which is a strong inhibitor of RmlB. After five batches (10 h), an average yield of 94% was obtained, analyzed by HPLC (with reference to **17**). Product isolation was performed as described above for **17** and gave 110 μmol (61 mg) of **13** (sodium salt) with a purity of 95% according to HPLC. The overall yield was 65 and 55% with reference to **17** and dTTP, respectively. The product was characterized as dTDP-2,6-dideoxy-α-D-*threo*-hexos-4-ulose by 1D/2D NMR spectroscopy in accordance with published data.[9] The successful deoxygenation at C-6 was indicated by the doublett of the three protons with a coupling constant of $^{3}J_{5,6}$ 6.5 Hz and the signal at 4.04 ppm for the H-5 quartett in the ^{1}H NMR spectrum; in addition, the upfield shift of the C-6 signal from 60.7 to 11.8 ppm in the ^{13}C NMR spectrum gave further evidence. The observed chemical shifts and heterocoupling constants in the ^{1}H NMR- ($^{3}J_{H\text{-}1,P}$ 6.4 and $J_{H\text{-}2ax,P}$ 3.0 Hz), ^{13}C NMR- ($^{2}J_{C\text{-}1,P}$ 5.9 and $^{3}J_{C\text{-}2,P}$ 7.4 Hz) and ^{31}P-(^{1}H-coupled) NMR-spectra ($^{2}J_{P,P}$ 19.8 Hz) were characteristic for dTDP-2,6-dideoxy-4-ketoglucose. The α configuration of the product was further indicated by the vicinal coupling constants $^{3}J_{1,2eq}$ 1.0 and $^{3}J_{1,2ax}$ 2.8 Hz, respectively. The signals at 93.4 and 209.0 ppm in the ^{13}C NMR spectrum were assigned to the hydrate- and keto-form at C-4, respectively. The negative ion mode ESI-MS spectrum of the product showed one intense peak in the mass region at m/z 551.0 corresponding to the $[M-Na]^{-}$ pseudo-molecular ion of dTDP-2,6-dideoxy-4-ketohexose. Further mass peaks for the keto- and hydrate-form of dTDP-2,6-dideoxy-4-ketohexose were observed at m/z 529.0 and m/z 547.0, respectively, in a ratio of ca. 4:1.

With the preparative synthesis of the biosynthetic intermediate **13** (Scheme 2), further access to dTDP-D- and L-2,6-dideoxyhexoses were possible. In our preliminary experiments, **13** was very unstable when incubated with RmlC alone. The enzymatic reaction produced an unstable compound which eliminated dTDP as detected by HPLC (data not shown). The observed instability of the reaction product may be due to the formation of a similar compound such as dTDP-3,4-diketo-2,6-dideoxyglucose (**12**) or its 2,3-enol, which was postulated as a product of the 2,3 dehydratase reaction with **11** as a substrate.[9] Recently the 3-D structure of two RmlC enzymes was resolved,[32–34] however, details on the reaction mechanism of RmlC and the chemical nature of the formed intermediates are yet not revealed.[35] In contrast, the biosynthetic intermediate **11** from the dTDP-L-rhamnose pathway was stable during incubation with RmlC (data not shown), which confirmed previously published data where the product of RmlC, dTDP-6-deoxy-L-*lyxo*-hexos-4-ulose, could be detected and characterized.[35,36] However, it could not be isolated in significant amounts due to its instability and the unfavorable equilibrium of the enzymatic reaction. We concluded that the product of the 3,5-epimerization of **13** is unstable and RmlD is needed to capture and stabilize the formed dTDP-2,6-dideoxy-L-hexose.

Enzymatic synthesis and isolation of dTDP-L-olivose.—The conversion of **13** by RmlC and RmlD (Scheme 3) yielded dTDP-L-olivose (**15**). The enzymatic reaction was performed in one batch with equimolar concentrations of **13** and NADPH. Fig. 2 illustrates the complete conversion within 5 h of incubation. Typically, the peak of the keto compound is very broad, whereas the product peak appears at a retention time of 13.57 min. Although dTDP was formed due to the instability of **15**, the synthesis yield was 77%, as analyzed by HPLC (with reference to **13**). Product isolation started with removal of proteins by ultrafiltration after dTDP was hydrolyzed by alkaline phosphatase. Two chromatographic steps (see above) yielded 51.1 μmol (28.3 mg, sodium salt) of **15** with an overall yield of 57%. The product was characterized as dTDP-

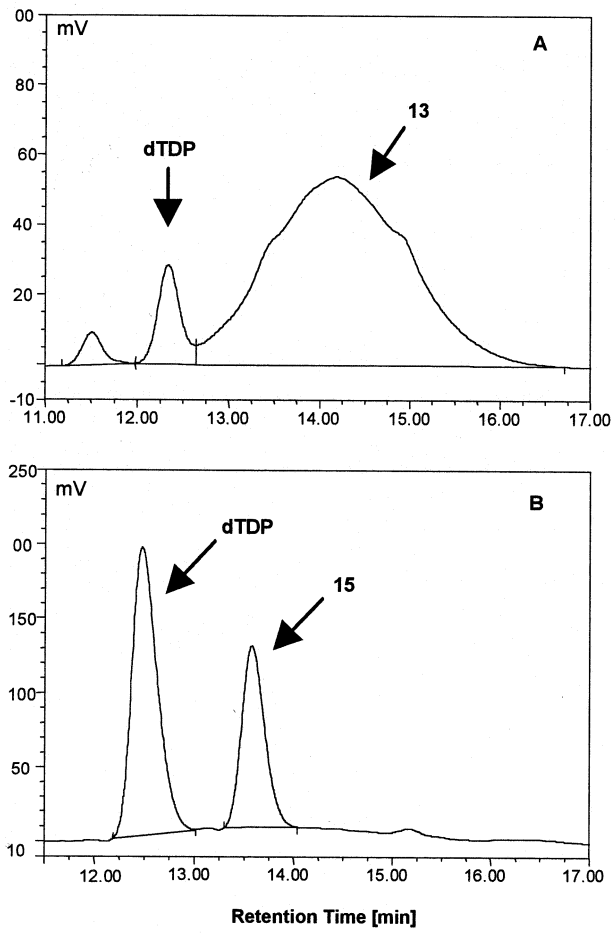


Fig. 2. HPLC chromatograms showing the complete conversion of **13** by RmlC and RmlD to yield **15**. (A) $t = 0$ h; (B) $t = 5$ h.

2,6-dideoxy-β-L-*arabino*-hexose by ^{1}H NMR spectroscopy. Characteristic signals and coupling constants were found for H-4 of the hexose moiety with $^3J_{4,5}$ 8.4 and $^3J_{3,4}$ 10.0 Hz, which are typical for the vicinal protons in the trans axial–axial position. In addition, the H-5 signal were assigned to the reduction of the carbonyl group at C-4 showing coupling constants of $^3J_{4,5}$ 8.4 and $^3J_{5,6}$ 6.0 Hz. The β configuration of the anomeric center of the hexose moiety was deduced from the hetero-coupling constant $J_{\text{H-1,P}}$ 8.4 Hz and the vicinal coupling constants of the trans axial–axial protons $^3J_{1,2ax}$ 10 Hz.

dTDP-L-olivose (**15**) is the first enzymatically synthesized nucleotide sugar, which is now available for in vitro studies of recombinant glycosyltransferases involved in antibiotic biosynthesis. The first target could be the oleandrosyltransferase OleG2 from the oleandomycin producer *Streptomyces antibioticus*. It was shown in biotransformation assays that the enzyme was also able to transfer L-olivose, a precursor of L-oleandrose.[10]

*Synthesis and isolation of dTDP-*D*-olivose* (**1**).—Chemical reduction of **13** with $NaBH_4$ gave a product peak with a yield of 56%, as analyzed by HPLC. However, the expected epimers **1** and **2** were not resolved by HPLC analysis. After gel filtration, 14.1 μmol (7.5 mg, monoprotonated form) of the product was obtained with an overall yield of 37%. The negative ion mode ESI-MS spectrum of the product showed one intense peak in the mass region at m/z 531.0 corresponding to the $[M - H]^-$ pseudo-molecular ion of dTDP-2,6-dideoxyhexose. Structural analysis by ^{1}H NMR spectroscopy revealed the epimer dTDP-2,6-dideoxy-α-D-*arabino*-hexose (**1**) as the main product. The typical signal at H-4 with coupling constants of $^3J_{4,5}$ 9.6 and $^3J_{4,3}$ 9.6 Hz was characteristic for the trans axial–axial arrangement of H-3, H-4, and H-5, which implies a preferred equatorial position of the hydroxyl group at C-4.

The product **1** could be very helpful for in vitro studies of recombinant glycosyltransferases, which were recently characterized in the biosynthetic gene clusters of mithramycin,[37,38] urdamycin A,[39,40] and landomycin A.[41]

Most interestingly, the incubation of **13** with RmlD over 4 days gave a product, which could unfortunately not be isolated as an intact compound (data not shown). However, preliminary analysis of the isolated product (5.8 mg) by ^{1}H NMR spectroscopy revealed dTDP and the glycal of D-oliose.

Conclusions.—The biochemical characterization of enzymes involved in the biosynthetic pathways of dTDP-activated 2,6-dideoxy-, 2,3,6-trideoxy-D- and L-hexoses can now be addressed by the preparative access to the 'branching-point' intermediate dTDP-2,6-dideoxy-4-ketoglucose (**13**). The presented synthesis strategy employing enzymes from the dTDP-L-rhamnose pathway also provides donor substrates for the in vitro characterization of glycosyltransferases involved in polyketide biosynthesis and may assist in the evaluation of the glycosylation potential of these enzymes to generate hybrid antibiotics.

3. Experimental

Materials and analytical methods.—Phosphoglucomutase (PGM) from rabbit muscle, alkaline phosphatase from calf intestine and inorganic pyrophosphatase (PPase) from yeast were purchased from Roche Diagnostics (Mannheim, Germany). Glucose-1,6-bisphosphate (Glc-1,6-P_2), dTTP and 2-deoxy-D-glucose-6-phosphate were supplied by Sigma (Deisenhofen, Germany). If not otherwise stated, all other material were from E. Merck (Darmstadt, Germany). Nucleotides and nucleotide sugars were analyzed by ion-pair reversed-phase HPLC using a KH_2PO_4/TBA–MeOH gradient system (column: Hypersil ODS-5μ, Chromatographie Service, Langerwehe, Germany).[42] Protein concentrations were determined by the Bradford method.[43] ^{1}H NMR spectra were recorded on a Bruker ARX 400 and Bruker AMX 300 spectrometer with DHO (4.80 ppm) as an internal reference in D_2O (300 K). ^{13}C NMR spectra were recorded on a Bruker AMX 300 spectrometer at 75.5 MHz in D_2O (300 K) and referenced with the methyl group of the TDP domain (12.0 ppm). ^{31}P NMR spectra were recorded on a Bruker AMX 300 spectrometer at 81.0 MHz in D_2O (300 K). Mass spectrometry was performed on a Finnigan LCQ using electrospray ionisation.

Bacterial strains and cultivation.—The bacterial strains expressing RmlA-D were kindly provided by Professor Dr W. Piepersberg (BUGH Wuppertal, Germany). The *rml*A—D genes from *Salmonella enterica* group B, serovar typhimurium (strain LT2), which encode α-D-glucose-1-phosphate deoxythymidylyltransferase (RmlA), dTDP-D-glucose-4,6-dehydratase (RmlB), dTDP-4-dehydrorhamnose 3,5-epimerase (RmlC) and dTDP-4-dehydrorhamnose reductase (RmlD) were cloned into the expression vector pT7-6 and expressed in *Escherichia coli* BL21 (DE3). The bacteria were grown aerobically at 30 °C in 500 mL conical flasks in Luria–Bertani broth (1% peptone, 0.5% yeast extract, 0.5% NaCl) containing 100 μg ampicillin/mL LB-medium. The cultivation was carried out at 30 °C with 5-L conical flasks, by shaking (140 rpm) until the culture reached an $OD_{600\ nm} = 1.0$. After addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG, 0.4 mM) cells were cultivated for further 60 min at 30 °C and 140 rpm. The cells were collected by centrifugation at 8000 rpm, washed three times with 50 mM Tris–HCl pH 8.0 and stored at −20 °C.

Partial purification of RmlA, RmlB, RmlC and RmlD.—RmlA was partially purified by anion-exchange chromatography on Q-Sepharose FF (Pharmacia, Freiburg, Germany, 2.6 × 34 cm, 4 mL min^{-1}) for the removal of phosphatases. A linear gradient of 0–500 mM NaCl in 20 mM Tris–HCl buffer, pH 8.0, containing 1 mM $MgCl_2$ and 22% (v/v) glycerol was applied. The fractions with the highest enzyme activity, as judged by the activity assay described by Ritter et al.,[44] were collected and adjusted to a pH-value of pH 8.0. The Q-Sepharose FF pool was concentrated in an ultrafiltration Amicon cell with a YM-10 ultrafiltration membrane (cut-off 10,000 Da, Amicon, Witten, Germany). The specific activity of RmlA of the crude extract (0.57 U mg^{-1} protein) was increased by the one-step purification to 4.8 U mg^{-1} protein. The purified enzyme solution was stored at −20 °C in 25% (v/v) glycerol. RmlB was partially purified by anion-exchange chromatography on Q-Sepharose FF (2.6 × 34 cm, 4 mL min^{-1}) using a linear gradient of 200–500 mM KCl in 50 mM Tris–HCl buffer, pH 7.5 (buffer A). The enzyme was further purified on Phenyl-Sepharose 6FF highsub (Pharmacia, Freiburg, Germany) by stepwise elution with 0.15, 0.08 and 0 M $(NH_4)_2SO_4$ in buffer A. A preparative gel filtration was subsequently performed on Superdex 200 prep grade (Pharmacia, Freiburg, Germany) by elution with 50 mM Tris–HCl buffer, pH 8.0, containing 150 mM KCl. The pool was concentrated by ultrafiltration as described above. The activity of RmlB was determined spectrophotometrically by the determination of formed dTDP-6-deoxy-4-keto-glucose at its characteristic absorption maximum at 318 nm in 0.1 M NaOH (molar absorption coefficient ($\varepsilon_{318\ nm}$) of 4800 L mol^{-1} cm^{-1}).[45] The specific activity of RmlB of the crude extract (1.15 U mg^{-1} protein) was increased by the three-step purification to 20.4 U mg^{-1} protein. The purified enzyme solution was stored at

−20 °C. RmlC and RmlD were partially purified by anion-exchange chromatography on Q-Sepharose FF (2.6 × 34 cm, 4 mL min^{-1}) using a linear gradient of 0–1 M NaCl in 20 mM Tris–HCl buffer, pH 8.0, containing 1 mM $MgCl_2$ and 22% (v/v) glycerol. The fractions containing RmlC or RmlD were analyzed by SDS-PAGE and pooled. The concentrated pools were stored at −20 °C in 33% (v/v) glycerol. Enzyme activities were determined as described elsewhere.[35]

Enzymatic synthesis, isolation and characterization of dTDP-2-deoxy-α-D-glucose (**17**).—The synthesis started from **18** by the combination of the enzymes PGM, RmlA and PPAse in a repetitive batch technique. In a stirred ultrafiltration cell (Amicon, Witten, Germany), 0.6 mmol **18** (184 mg) and 0.1 mmol dTTP (61.4 mg) were dissolved in 50 mM Tris–HCl buffer, pH 8.0, containing 5 mM $MgCl_2$ and 3 μM Glc-1,6-P_2. After the addition of 10 U mL^{-1} PPase, 0.6 U mL^{-1} PGM, and 3 U mL^{-1} RmlA, the reaction mixture (50 mL final volume) was incubated at 30 °C for 3 h. After complete conversion of dTTP, as analyzed by HPLC, the product solution was separated from the enzymes by ultrafiltration to yield a residual volume of 5 mL. A second batch was started by the addition of 45 mL of fresh substrate solution (containing 0.6 mmol **18** and 0.1 mmol dTTP) and treated as described before. After five batches, **17** was obtained with an average yield of 86% (referring to dTTP). Alkaline phosphatase (EC 3.1.3.1) was added to the pooled product solution in order to hydrolyze dTDP as a contaminant of commercial dTTP. After ultrafiltration the product solution was applied to an anion-exchange chromatography with Dowex 1 × 2, Cl^--form (2.6 × 33 cm) using a linear gradient of 0–600 mM NaCl in distilled water (flow rate of 5 mL min^{-1}). The fractions containing **17** (analyzed by HPLC) were pooled and concentrated by in vacuo evaporation to 20 ml. Desalting of the product was carried out at 4 °C on Sephadex G-10 (5.0 × 88 cm, 2 mL min^{-1}). The fractions were pooled and stored at −72 °C. After lyophilization, **17** was obtained as white powder (60.8 μmol, 36 mg, di-sodium salt) with an overall yield of 61% (referring to dTTP). HPLC analysis revealed a purity of 94% with 6% dTDP.

^{1}H NMR (300 MHz): 2-deoxysugar domain δ 5.69 (br dd, 1 H $^3J_{1,P}$ 6.8, $J_{1,2ax}$ 3.0 Hz, H-1), 3.98 (ddd, 1 H, $J_{2ax,3}$ 11.8, $J_{3,4}$ 9.5, $J_{2eq,3}$ 4.6 Hz, H-3), 3.88–3.81 (m, 2 H, H-6a,b), 3.77 (m, 1 H, H-5), 3.40 (t, 1 H, $J_{4,5}$ 9.5 Hz, H-4), 2.25 (ddd, 1 H, $J_{2eq,2ax}$ 13.2, $J_{1,2eq}$ 0.8 Hz, H-2eq), 1.70 (dddd, 1 H, $^4J_{2ax,P}$ 3.0 Hz, H-2ax); dTDP domain δ 7.57 (s, 1 H, H-6″), 6.32 (dd, 1 H, $J_{1',2a'}$ 7.1, $J_{1',2b'}$ 6.7 Hz, H-1′), 4.60 (m, 1 H, H-3′), 4.14–4.06 (m, 3 H, H-4′, H-5a′, H-5b′), 2.33 (m, 2 H, H-2a′, H-2b′), 1.91 (s, 3 H, CH_3-5″); ^{13}C NMR (75.5 MHz): 2-deoxysugar domain δ 37.7 (d, $^3J_{C-2,P}$ 7.5 Hz, C-2), 60.7 (C-6), 68.0 (C-3), 70.8 (C-5), 73.8 (C-4), 95.0 (d, $^2J_{C-1,P}$ 6.0 Hz, C-1); dTDP domain δ 12.0 (CH_3), 38.8 (C-2′), 65.6 (d, $^2J_{C-5',P}$ 6.0 Hz, C-5′), 71.2 (C-3′), 85.2 (C-1′), 85.5 (d, $^3J_{C-4',P}$ 9.0 Hz, C-4′), 112.0 (C-5″), 137.5 (C-6″), 152.5 (C-2″), 167.5 (C-4″); ^{31}P NMR (81.0 MHz): δ −10.18 (d, J 20.3 Hz), -12.19 (d, J 20.3 Hz).

Enzymatic synthesis, isolation and characterization of dTDP-2,6-dideoxy-4-ketoglucose (**13**).—Following the same repetitive batch procedure **13** was prepared from **17**, which was synthesized as described before. To a 50 mL batch containing 168 μmol **17** in 50 mM Tris–HCl, pH 8.0 RmlB (10 U mL^{-1}) and alkaline phosphatase (2 U mL^{-1}) were added and incubated at 30 °C. The latter suppressed the strong competitive inhibition of the RmlB by dTDP (K_i(dTDP) = 2.5 μM).[21] After 2 h **17** was completely converted as analyzed by HPLC. The product solution was filtrated and a second batch was started by the addition of **17** as described above. After five batches **13** was obtained with an average yield of 94% (with reference to **17**). The isolation of **13** was performed by ultrafiltration, anion-exchange chromatography, desalting and lyophilization as described above for **17**. dTDP-2,6-dideoxy-4-keto-D-glucose (**13**) was obtained as white powder (61 mg, 110 μmol, monosodium salt) with an overall yield of 65% (referring to **17**). HPLC analysis revealed a purity of 95% with 2% dTMP and 3% dTDP. Analyses by ESI-MS (negative ion mode) and ^{1}H, ^{13}C, ^{31}P NMR-data confirmed the integrity of **13** and corresponded exactly to those previously reported.[9]

^{1}H NMR (300 MHz): 2,6-dideoxysugar domain δ 5.61 (ddd, 1 H, $^3J_{1,P}$ 6.4, $J_{1,2ax}$ 2.8, $J_{1,2eq}$ 1.0 Hz, H-1), 4.04 (q, 1 H, $J_{5,6}$ 6.5 Hz, H-5), 4.00 (dd, 1 H, $J_{2ax,3}$ 11.8, $J_{2eq,3}$ 4.9 Hz, H-3), 2.14 (ddd, 1 H, $J_{2eq,2ax}$ 12.9 Hz, H-2eq), 1.81 (dddd, 1 H, $J_{2ax,P}$ 3.0 Hz, H-2ax), 1.20 (d, 3 H, H-6); dTDP domain δ 7.69 (s, 1 H, H-6″), 6.33 (dd, 1 H, $J_{1',2a'}$ 7.1, $J_{1',2b'}$ 6.9 Hz, H-1′), 4.60 (m, 1 H, H-3′), 4.14–4.06 (m, 3 H, H-4′, H-5a′, H-5b′), 2.35 (m, 2 H, H-2a′, H-2b′), 1.92 (s, 3 H, CH_3-5″); ^{13}C NMR (75.5 MHz): 2,6-dideoxysugar domain δ 11.8 (C-6), 36.2 (d, $^3J_{C\text{-}2,P}$ 7.4 Hz, C-2), 68.4 (C-3), 70.4 (C-5), 93.4 (C-4), 94.9 (d, $^2J_{C\text{-}1,P}$ 5.9 Hz, C-1); dTDP domain δ 12.0 (CH_3), 39.0 (C-2′), 65.8 (d, $^2J_{C\text{-}5',P}$ 6.0 Hz, C-5′), 71.3 (C-3′), 85.3 (C-1′), 85.7 (d, $^3J_{C\text{-}4',P}$ 9.2 Hz, C-4′), 112.1 (C-5″), 137.7 (C-6″), 152.1 (C-2″), 167.0 (C-4″); ^{31}P NMR (81.0 MHz) δ −10.34 (d, J 19.8 Hz), −12.34 (d, J 19.8 Hz). ESI-MS (negative ion mode): m/z 529 [51%, $M^{-2}+H^+$], 547 [12%, M^{-2} + water + H^+], 551 [100%, $M^{-2}+Na^+$], calculated m/z 552.3 [$M^{-2}+Na^+$].

*Enzymatic synthesis, isolation and characterization of dTDP-*L*-olivose* (**15**).—In a total reaction volume of 44 mL **13** (90 μmol, 51.7 mg) and NADPH (90 μmol, 68.7 mg) were dissolved in 50 mM Tris–HCl, pH 8.0. The reaction was started by the addition of RmlC (24 U) and RmlD (189 U). The formation of **15** was followed by HPLC. After incubation for 5 h at 30 °C, alkaline phosphatase (2 U mL^{-1}) was added to hydrolyze dTDP which was formed as hydrolysis product during the reaction. After incubation for 2 h, all enzymes were subsequently separated from the product solution by ultrafiltration. Production isolation was accomplished by anion-exchange chromatography on Dowex 1 × 2 (Cl^--form) with a linear gradient of 0–800 mM NaCl in distilled water (flow rate 5 mL min^{-1}). The fractions containing **15** were pooled and concentrated by in vacuo evaporation. Desalting was carried out at 4 °C as described above. After lyophilization dTDP-β-L-olivose was obtained as a white powder (28.3 mg, 49.1 μmol, monosodium salt) with an overall yield of 57% (with reference to **13**).

^{1}H NMR (400 MHz): 2,6-dideoxysugar domain δ 5.27 (ddd, 1 H, $J_{1,2ax}$ 10.0, $J_{1,P}$ 8.4, $J_{1,2eq}$ 2.0 Hz, H-1), 4.63 (m, 1 H, H-3), 3.46 (dq, 1 H, $J_{4,5}$ 8.4, $J_{5,6}$ 6.0 Hz, H-5), 3.07 (dd, 1 H, $J_{3,4}$ 10.0 Hz, H-4), 1.96–1.92 (m, 1 H, H-2eq), 1.61 (ddd, 1 H, $J_{2ax,2eq}$ 12.2, $J_{2ax,3}$ 11.8 Hz, H-2ax), 1.31 (d, 3 H, H-6); dTDP domain δ 7.77 (s, 1 H, H-6″), 6.38 (t, 1 H, $J_{1',2a'}$ 6.9, $J_{1',2b'}$ 6.9 Hz, H-1′), 4.63 (m, 1 H, H-3′), 4.22–4.15 (m, 3 H, H-4′, H-5a′, H-5b′), 2.43–2.36 (m, 2 H, H-2a′, H-2b′), 1.95 (s, 3 H, CH_3-5″).

*Synthesis, isolation and characterization of dTDP-*D*-olivose* (**1**).—The 4-keto compound **13** (20.41 mg, 36 μmol) was dissolved in 2 ml distilled water and three aliquots of $NaBH_4$ (2.02 mg, 53.3 μmol) were added at a pH of 7.5 during a reaction period of 5 h. Complete conversion of **13** was analyzed by HPLC. The product yield was 56% (with reference to **13**), however, the expected epimers **1** and **2** could not be separated by HPLC analysis. Alkaline phosphatase (10 U mL^{-1}) was added to hydrolyze dTDP which was formed in large amounts during reduction. The product was purified by gel filtration on Sephadex G-10 as described above. After lyophilization the product was obtained with an overall yield of 37% (7.5 mg, 14.1 μmol, monoprotonated compounds) and analyzed by ^{1}H NMR. Although the formation of the epimers **1** and **2** was expected, the NMR data for **2** could not be extracted from the spectra of the product mixture. The following NMR data refer to compound **1**.

^{1}H NMR (400 MHz) 2,6-dideoxysugar domain δ 5.63 (ddd, 1 H, $J_{1,P}$ 6.0, $J_{1,2ax}$ 2.4, $J_{1,2eq}$ 1.2 Hz, H-1), 3.98–3.90 (m, 2 H, H-3, H-5), 3.07 (t, 1 H, $J_{3,4}$ 9.6, $J_{4,5}$ 9.6 Hz, H-4), 2.28 (ddd, 1 H, $J_{2eq,2ax}$ 13.6, $J_{2eq,3}$ 4.6 Hz, H-2eq), 1.72 (dddd, 1 H, $J_{2ax,3}$ 11.6, $J_{2ax,P}$ 3.0 Hz, H-2ax), 1.27 (d, 3 H, $J_{5,6}$ 6.4 Hz, H-6); dTDP domain δ 7.76 (s, 1 H, 6″-H), 6.35 (t, 1 H, $J_{1',2a'}$ 7.0, $J_{1',2b'}$ 7.0 Hz, H-1′), 4.63 (m, 1 H, 3′-H), 4.20–4.15 (m, 3 H, H-4′, H-5a′, H-5b′), 2.40–2.34 (m, 2 H, H-2a′, H-2b′), 1.93 (s, 3 H, CH_3-5″). ESI-MS (negative ion mode): m/z 531 [100%, $M^{-2}+H^+$], 553 [5%, $M^{-2}+Na^+$]. [$M^{-2}+H^+$] calculated m/z 530.31.

Acknowledgements

The authors thank Professor Dr W. Piepersberg (BUGH Wuppertal) for providing the

recombinant *E. coli* strains expressing RmlA, RmlB, RmlC and RmlD, Dr J.C. Namyslo (TU Clausthal–Zellerfeld) for his excellent help in NMR analysis, Dr Rettberg (University Göttingen) for ESI–MS analysis. The authors thank Professor Dr P.F. Leadlay (Cambridge University) and Dr Martin Hein for critical reading of the manuscript. Financial supports by EU the grants 'Hyglide' (Contract No. ERBBIO 4 CT 960080) and 'GENOVA' (Contract No. QLK3-999-00095) to L.E. is gratefully acknowledged.

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