An Enzyme Module System for the Synthesis of dTDP-activated Deoxysugars from dTMP and Sucrose

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*A flexible enzyme module system is presented that allows preparative access to important dTDP-activated deoxyhexoses from dTMP and sucrose. The strategic combination of the recombinant enzymes dTMP-kinase and sucrose synthase (SuSy), and the enzymes RmlB (4,6-dehydratase), RmlC (3,5-epimerase) and RmlD (4-ketoreductase) from the biosynthetic pathway of dTDP-β-L-rhamnose was optimized. The SuSy module (dTMP-kinase, SuSy, ±RmlB) yielded the precursor dTDP-α-D-glucose (**2**) or the biosynthetic intermediate dTDP-6-deoxy-4-keto-α-D-glucose (**3**) on a 0.2–0.6 g scale with overall yields of 62% and 72%, respectively. A two-step strategy in which the SuSy module was followed by the deoxysugar module (RmlC and RmlD) resulted in the synthesis of dTDP-β-L-rhamnose (**4**; 24.1 μmol, overall yield: 35.9%). Substitution of RmlC by DnmU from the dTDP-β-L-daunosamine pathway of* Streptomyces peucetius *in this module demonstrated that DnmU acts in vitro as a 3,5-epimerase with **3** as substrate to yield **4** (32.2 μmol, overall yield: 44.7%). Chemical reduction of **3** with $NaBH_4$ gave a mixture of the C-4 epimers dTDP-α-D-quinovose (**6**) and dTDP-α-D-fucose (**7**) in a ratio of 2:1. In summary, the modular character of the presented enzyme system provides valuable compounds for the biochemical characterization of deoxysugar pathways playing a major role in microbial producers of antibiotic and antitumour agents.*

Introduction

The D- and L-deoxyhexoses are important carbohydrate components that mediate the biological functions of glycoconjugates in animals, plants and microorganisms.[1–3] The structural diversity of these glycoconjugates is determined by the distinct biosynthetic pathways of dTDP-, CDP- and GDP-activated sugars and the substrate specificity of glycosyltransferases. The biosyntheses of polyketide antibiotics include deoxysugar pathways of dTDP-activated deoxyhexoses modified at C-2, C-3, C-4 and C-6 by deoxygenation, epimerization, amination and C- and O-methylation. These complex D- and L-deoxyhexoses are important for the antibiotic and antitumour bioactivity of polyketides.[4–11] Biochemical studies have revealed that the common precursor dTDP-α-D-glucose (dTDP-Glc; **2**) is formed by conversion of α-D-glucose-1-phosphate (**1**) and dTTP catalyzed by dTDP-Glc pyrophosphorylases (dTDP-Glc PP, EC 2.7.7.24; Scheme 1). An important feature of all these biosynthetic pathways is the occurrence of the central intermediate dTDP-4-keto-6-deoxyglucose (**3**), which is formed by the dTDP-Glc-4,6-dehydratase (EC 4.2.1.46) by oxidation at C-4 and subsequent β-elimination of water at C-6.[2,12–18] The central role of **3** is emphasized in studies on the biosynthesis of dTDP-activated 6-deoxysugars, such as dTDP-β-L-rhamnose (**4**),[17,19,20] dTDP-6-deoxy-β-L-talose (dTDP-β-L-pneumose; **5**),[21] dTDP-α-D-quinovose (**6**)[22] and dTDP-α-D-fucose (**7**; Scheme 1).[23] In addition, **3** represents the biosynthetic gateway to complex dTDP-deoxyhexoses (Scheme 2). Isomerization of **3** at C-4 and C-3 with subsequent amination of the resulting C-3 carbonyl affords dTDP-3-amino-3,6-dideoxy-α-D-glucose (**8**), the precursor of dTDP-D-mycaminose and dTDP-D-desosamine.[24–26] The deoxygenation of **3** at C-2 and stereospecific reduction of the C-3 carbonyl produces dTDP-2,6-dideoxy-4-keto-D-glucose (**9**) or -D-allose (**10**). Both are important biosynthetic intermediates for the synthesis of dTDP-2,6-dideoxyhexoses.[27–31] The deoxygenation of **3** at C-2 and stereospecific amination of the C-3 carbonyl results in the dTDP-3-amino-2,3,6-trideoxyhexoses **11** and **12**, the precursors of TDP-L-epivancosamine and dTDP-L-daunosamine, respectively.[32–34] Some of these studies have revealed the stereochemistry of enzymatic reactions in deoxysugar pathways; however, the complete order of the biosynthetic steps, as well as the in vitro kinetics and the substrate spectra of the individual enzymes, still remain to be determined. These in vitro biochemical studies are important for in depth understanding and engineering of these metabolic deoxysugar pathways to obtain novel antitumour and antibiotic drugs by glycosylation engineering.[24,32,35–37] However, preparative access to

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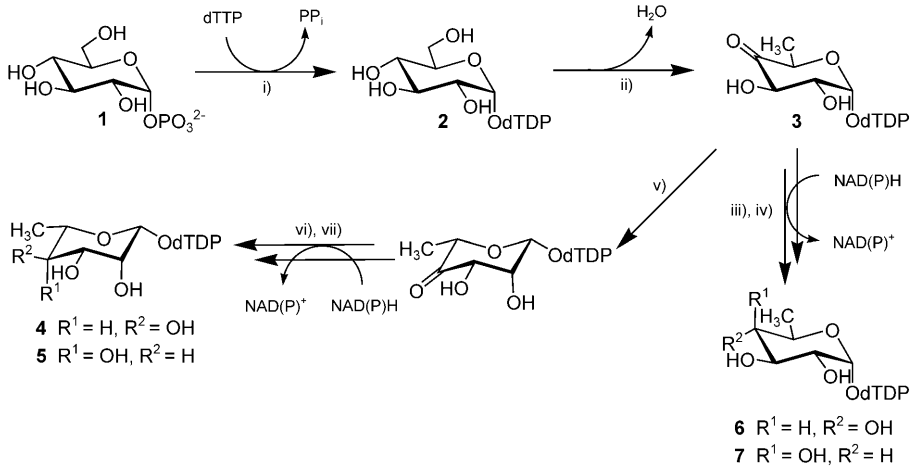
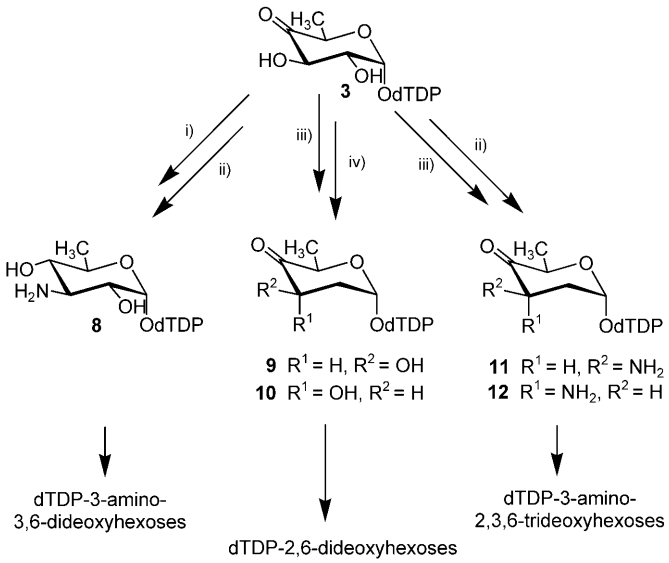


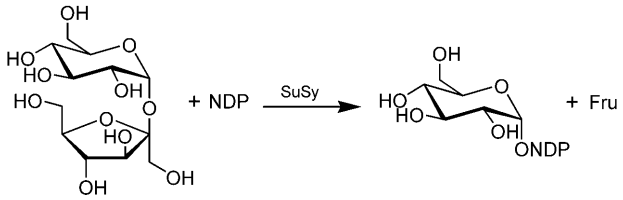
Scheme 1. Biosynthetic pathway for dTDP-Glc (**2**) and dTDP-4-keto-6-deoxy-glc (**3**): i) RmlA, dTDP-Glc pyrophosphorylase (dTDP-Glc PP, EC 2.7.7.24), ii) RmlB, dTDP-Glc-4,6-dehydratase (EC 4.2.1.46), iii) dTDP-6-deoxyhexosyl-4-ulose reductase, iv) dTDP-6-deoxyhexosyl-4-ulose reductase Fcd, v) dTDP-4-dehydrorhamnose 3,5-epimerase RmlC (EC 5.1.3.13), vi) dTDP-4-dehydrorhamnose-reductase RmlD (EC 1.1.1.133), vii) dTDP-6-deoxy-L-talose dehydrogenase Tll.

first nucleotide sugar in a continuous mode in an enzyme membrane reactor by use of SuSy.[46] 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 **3**.[18] The gram-scale synthesis of this central intermediate was accomplished in a fed-batch mode with account being taken of the fact that dTDP is a very strong competitive inhibitor ($K_i = 2.5$ μmol) of RmlB with respect to the substrate dTDP-Glc.[47]

Another method for the production of **3** was established by



Scheme 2. Biosynthetic pathways of dTDP-activated deoxyhexoses: i) 3,4-isomerase, ii) 3-keto transaminases, iii) 2,3-dehydratases, iv) 3-keto reductases; each arrow indicates one enzymatic reaction.



Scheme 3. Synthesis of nucleotide sugars from sucrose and nucleoside diphosphates (NDPs) by employment of sucrose synthase (EC 2.4.1.13; SuSy).[42]

precursors and central intermediates of dTDP-deoxyhexose pathways, as well as the availability of donor substrates of glycosyltransferases, is still limited.

In this context, the aim of our work is to provide precursors, central intermediates of dTDP-deoxyhexose pathways and donor substrates of glycosyltransferases for biochemical studies.[38–41] In contrast to the pathways described, we followed alternative synthetic routes employing sucrose synthase (EC 2.4.1.13; SuSy) from rice grains[42] and recombinant SuSy1 from potato[40,43–45] in order to establish novel means of access to nucleotide sugars from sucrose and nucleoside diphosphates (NDPs; Scheme 3). In this way we synthesized **2** as the using dTMP-kinase, actetate kinase, RmlA and RmlB.[48] Oh et al. produced **3** in a one-pot synthesis (0.96 g, 81% overall yield based on dTMP), but use of high substrate concentrations (100 mM dTMP and 400 mM of **1**) resulted in low conversion yields (30%) due to inhibition. Furthermore, RmlA was difficult to express because of the formation of large amounts of inclusion bodies.[48]

In our approach, using the fed batch technique and recombinant SuSy from potato instead of RmlA, we were able to circumvent the reported drawbacks and to provide a synthesis based on sucrose as starting material. Thus we established the first economic syntheses of different NDP-α-D-glucoses (N = U, dU, C, A) from nucleoside monophosphates (NMP) and sucrose through combinations of monophosphate kinase and myokinase, respectively, and SuSy.[40,49]

In this paper, we report on a flexible enzyme module system starting from dTMP and sucrose for the preparative (chemo)enzymatic synthesis of the precursor **2**, the intermediate **3**, dTDP-β-L-rhamnose (**4**), dTDP-α-D-quinovose (**6**) and dTDP-α-D-fucose (**7**). The enzyme module system also allows the biochemical characterization of enzymes from deoxysugar pathways.

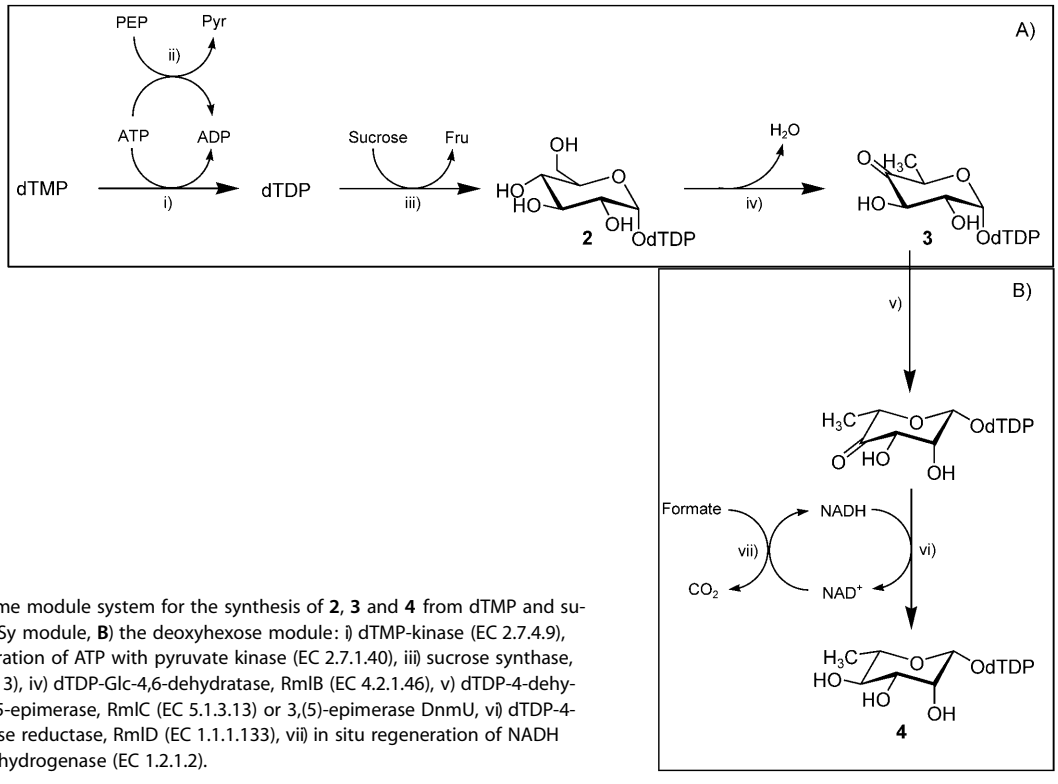
Results and Discussion

Scheme 4 depicts the overall strategy to obtain dTDP-6-deoxyhexoses by enzymatic synthesis from dTMP and sucrose. In the so-called SuSy module (Scheme 4A) recombinant dTMP-kinase[50,51] (EC 2.7.4.9) from yeast, recombinant SuSy1[45] from potato and recombinant dTDP-Glc-4,6-dehydratases (EC 4.2.1.46, RmlB) are combined. The main advantage is that dTMP is a less expensive substrate from which to start a preparative synthesis of **2** and **3**, respectively, together with the in situ regeneration of the cofactor ATP.

The K_m values of dTMP-kinase for the substrates dTMP and ATP were reported as 9 μM and 190 μM, respectively.[51] Kinetic analysis of SuSy1 revealed a K_m of 12 μM for dTDP and a V_{max} of 0.35 U mg^{-1}, which corresponds to 14% of the reaction rate with the natural substrate UDP and a 214 times lower catalytic efficiency.[45] However, a remarkable advantage of dTDP as substrate is that the activity of SuSy is not inhibited by higher substrate concentrations. In contrast, we identified dTMP as a competitive inhibitor for the substrate dTDP with a K_i of 21.3 mM. In the synthesis of **2**, however, SuSy1 activity was not affected by starting the first batch with 4 mM dTMP and the following nine repetitive batches with an average concentration of 3.2 mM dTMP. The utilization of all enzymes in a repetitive batch mode gives a further improvement with respect to the product-specific enzyme consumption and the space-time yield. In a repetitive batch mode, all enzymes are reused through the removal of 80% of the product solution by ultrafiltration and subsequent addition of fresh substrate solution to start the next batch. After ten batches the average synthesis yield for **2** was 94% (1.54 mmol) with reference to dTMP (Figure 1). Product isolation gave 1.02 mmol (618 mg) of **2** with an overall yield of 62%. The space-time yield was determined as 3.1 g L^{-1} d^{-1} with a product-specific enzyme consumption of 106 U g^{-1} for SuSy1 and 53 U g^{-1} for dTMP-kinase. Characterization by ^{1}H and ^{13}C NMR confirmed the integrity of **2** by comparison with published data.[46]

dTDP-Glc-4,6-dehydratase (RmlB) was further included in the SuSy module for the one-pot synthesis of **3** (Scheme 4A). Starting the synthesis from dTMP was highly beneficial, because RmlB is severely inhibited by dTDP. The irreversible reaction of RmlB also increased the rate of product formation. As a consequence, the efficiency of the synthesis could be optimized by periodic feeding of dTMP in a repetitive batch synthesis. After five batches **3** was obtained in a yield of 97% (0.43 mmol, 253 mg) with reference to dTMP. After product isolation, 0.31 mmol of **3** (183.5 mg, 72.2% overall yield) was obtained. The space-time yield for the enzymatic reaction was 8.3 g L^{-1} × d^{-1} with a product-specific enzyme consumption of 119 U g^{-1} for SuSy, 40 U g^{-1} for dTMP kinase and 196 U g^{-1} for RmlB. Characterization by ^{1}H and ^{13}C NMR confirmed the integrity of **3** by comparison with published data.[18]

In a preliminary experiment, the SuSy module was combined with the enzymes RmlC and RmlD (deoxysugar module) for a one-pot synthesis of **4** (Scheme 4), resulting in a yield of only 32%. Feed-back inhibition by **4** had previously been described for RmlA, but not for the other Rml-pathway enzymes.[52] A closer analysis of the combined reaction of dTMP-kinase and SuSy1 revealed a 50% lower production rate for **2** at 1.27 mM of **4**. A two-step strategy for the enzymatic synthesis of **4**—in



Scheme 4. Enzyme module system for the synthesis of **2**, **3** and **4** from dTMP and sucrose. **A**) The SuSy module, **B**) the deoxyhexose module: i) dTMP-kinase (EC 2.7.4.9), ii) in situ regeneration of ATP with pyruvate kinase (EC 2.7.1.40), iii) sucrose synthase, SuSy1 (EC 2.4.1.13), iv) dTDP-Glc-4,6-dehydratase, RmlB (EC 4.2.1.46), v) dTDP-4-dehydrorhamnose 3,5-epimerase, RmlC (EC 5.1.3.13) or 3,(5)-epimerase DnmU, vi) dTDP-4-dehydrorhamnose reductase, RmlD (EC 1.1.1.133), vii) in situ regeneration of NADH with formate dehydrogenase (EC 1.2.1.2).

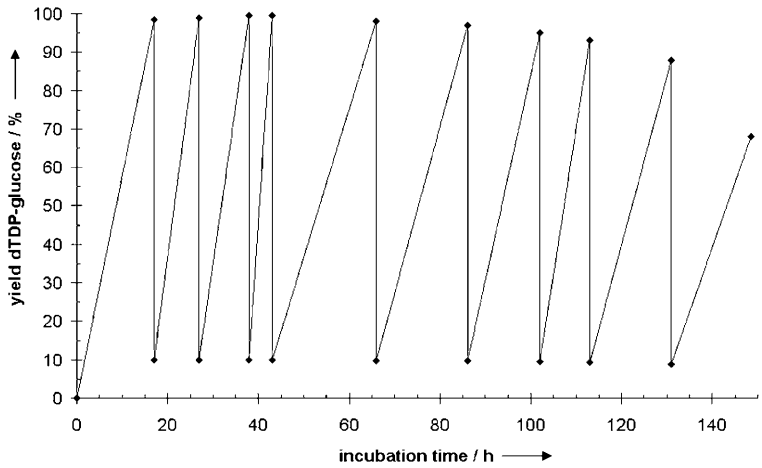
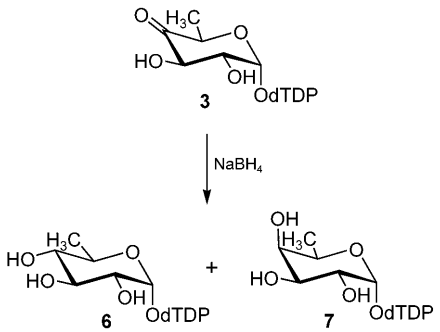


Figure 1. Conversion levels in ten repetitive batches over 150 h giving **2** with an average yield of 94%.

which the SuSy module (Scheme 4A) and the deoxysugar module (Scheme 4B) were utilized separately—was therefore chosen. The first step gave **3** as described for the SuSy module. The second step comprised the synthesis of **4** from **3** with in situ regeneration of the cofactor NADH. In this module an average yield of 94% (63.2 μmol, 37.4 mg) for **4** over three repetitive batches was reached. After product isolation, **4** (24.1 μmol, 14.3 mg) was obtained in an overall yield of 35.9% with reference to dTMP. The loss of product is due to very strict pooling of fractions from a desalting step on a Sephadex G10 column to obtain highly pure (99%) product for characterization by ^{1}H NMR. In a previous report, a higher scale (284 μmol) with a yield of 62% was obtained for **4**, but the synthesis here started from the pure, commercially available dTDP-Glc.[17]

In order to extend the availability of dTDP-6-deoxyhexoses the central intermediate **3** was further chemically reduced with $NaBH_4$ (Scheme 5). The reaction proceeded to almost complete conversion of **3** (Figure 2). A mixture of the C-4 epimers dTDP-α-D-quinovose (**6**) and dTDP-α-D-fucose (**7**) was obtained in a ratio of 2:1 and with a yield of 91% (11.4 μmol, 6.8 mg). The products were isolated as a mixture of the epimers by gel filtration on Sephadex G15 in 86% overall yield (10.7 μmol, 6.4 mg) and analyzed by ^{1}H NMR. Characterization confirmed the integrity of **7** by comparison with published data.[23]



Scheme 5. Chemical reduction of **3** with $NaBH_4$ in aqueous solution.

The modular character of our reaction scheme (Scheme 4) offers advanced applications for combinatorial biocatalysis. Starting with **3** in the deoxysugar module it is possible to characterize and use single enzymes from different deoxysugar pathways for preparative synthesis. A first example is presented here, in a test of the in vitro synthetic potential of recombinant DnmU from the dTDP-L-daunosamine pathway of *Streptomyces peucetius* (daunorubicin producer).[53] Complementation analyses of mutant strains indicated that DnmU functions as a 3,5-epimerase of **3** or a later intermediate,[53] but other studies were able to demonstrate clearly that 2-deoxygenation precedes epimerization and modification steps,[28,30] so the function of DnmU as a 5-epimerase with compound **12** seems reasonable. A similar role for EvaD had already been demonstrated in the pathway of dTDP-L-epivancosamine, where compound **11** was identified as the second central intermediate of dTDP-3-amino-2,3,6-trideoxyhexoses (Scheme 2).[34] Furthermore, not only a 5-epimerization reaction with the natural substrate dTDP-3-amino-3-methyl-2,6-dideoxyglucose was indicated, but also a 3,5-epimerase activity with **3**.[54] However, the 5-epimerization activity was 200 times higher than the 3,5-epimerization activity of EvaD because of a change in the enzyme's active site in relation to RmlC.[55,56]

The in vitro potential of DnmU to epimerize the first central intermediate **3** was tested in our enzyme module system by replacing the 3,5-epimerase RmlC (Scheme 4B). An almost identical yield of **4** (97% yield, 65.2 μmol) was obtained with an identical amount of enzyme. The isolated product (47.9%, 32.2 μmol, 19.1 mg) was confirmed as dTDP-β-L-rhamnose by ^{1}H NMR. Our result demonstrates the in vitro activity of DnmU as a dTDP-4-keto-6-deoxyglucose 3,5-epimerase for the first time. However, in vivo studies demonstrated that DnmU could complement EryBVII, the proposed (3),5-epimerase from the dTDP-L-mycarose pathway of *Saccharopolyspora erythraea* (erythromycin A producer).[57] In addition, TylK, the homologous enzyme to EryBVII in the dTDP-L-mycarose pathway of *Streptomyces fradiae* (tylosin producer),[58] was identified in vitro as a 5-epimerase.[9,30,59] In conclusion, these results suggest that in vivo DnmU catalyzes only the C-5 epimerization, as the enzymes TylK and EryBVII do. However, the in vitro function of EryBVII still has to be determined and it cannot be ruled out that DnmU might also act in vitro, like EvaD with its natural substrate, as a 5-epimerase in a dTDP-L-mycarose biochemical background.

Conclusion

In summary, our results demonstrate the potential of our combinatorial biocatalysis approach to the synthesis of dTDP-deoxysugars from dTMP and sucrose on preparative scales. The modular character of our enzyme system further allows the

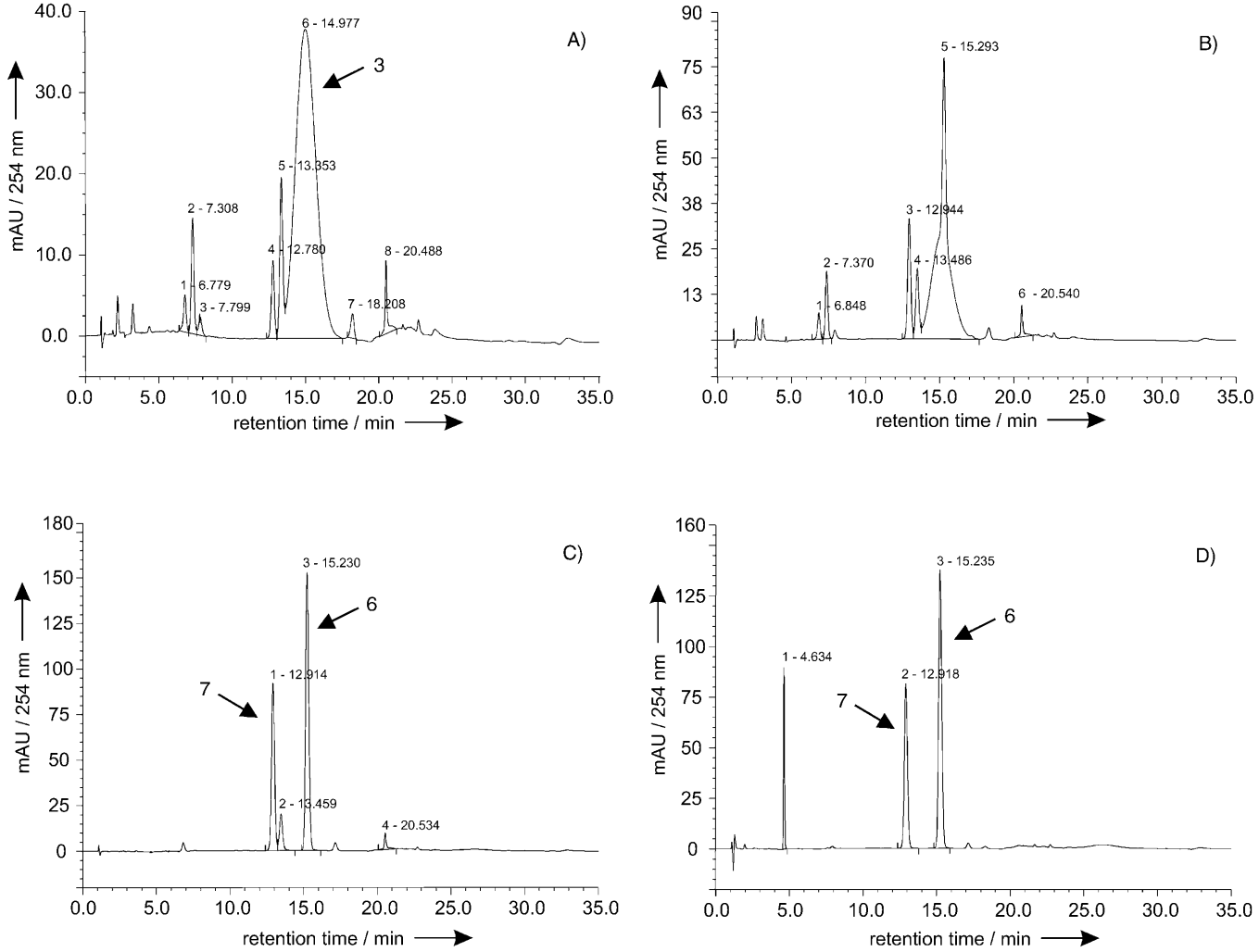


Figure 2. Time course of the chemical reduction of **3** with $NaBH_4$ to yield a mixture of the 4-epimers **6** and **7**: **A**) **3** before addition of $NaBH_4$, **B**) after the first addition of $NaBH_4$ (0.2 mM), **C**) after the third addition of $NaBH_4$ (0.2 mM)—products **6** and **7** are formed, the peak at 13.459 min is dTDP, which disappears in **D**, **D**) after the addition of alkaline phosphatase—the peak at 4.634 min is thymidine.

replacement and characterization of dTDP-deoxysugar pathway enzymes from microbial drug (antibiotics, antitumour agents) producers. Work to incorporate recombinant macrolide glycosyltransferases for the characterization of their donor and acceptor substrate spectra is also in progress. This enzyme module system would then facilitate the in situ regeneration of dTDP-activated deoxysugars from sucrose and dTDP with sucrose synthase, similarly to the in situ regeneration of UDP-Gal.[60–62] In addition, the in vitro characterization of macrolide glycosyltransferases together with deoxysugar pathway enzymes should assist current biotransformation approaches[24,32,37,63–65] for the production of novel antibiotics by glycosylation engineering.

Experimental Section

Materials and analytical methods: Pyruvate kinase (PK), alkaline phosphatase (AP), formate dehydrogenase (FDH), ATP (Na_2-salt), phosphoenolpyruvate (PEP, $Na{\cdot}H_2O$) and NADH were purchased from Roche Diagnostics (Mannheim, Germany). Thymidine 5′-monophosphate (dTMP, Na_2 salt), thymidine 5′-diphosphate (dTDP, Na_2 salt) and thymidine 5′-diphosphate-α-D-glucose (dTDP-Glc, Na_2 salt) were supplied by Sigma (Deisenhofen, Germany). If not otherwise stated all other material were from Merck (Darmstadt, Germany). Nucleotides and nucleotide sugars were analyzed by ion-pair reversed-phase HPLC with a KH_2PO_4/TBA-methanol gradient system (column: Hypersil ODS-5μ, Chromatographie Service, Langerwehe, Germany) with UV detection at 254 nm.[66] Protein concentrations were determined by the Bradford method.[67] ^1H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 spectrometer.

Recombinant enzymes: The production and purification of recombinant sucrose synthase1 (SuSy1) from potato, expressed in *Saccharomyces cerevisiae*, was performed as described previously.[45] The activity of SuSy1 was assayed as reported earlier.[68] Strains of *Escherichia coli* BL21(DE3) harbouring the vectors pTHK1(pJC20) and pTHK1(pJC20)His_6-C for the expression of the *thk1* gene encoding dTMP-kinase from *Saccharomyces cerevisiae* were kindly provided by Dr. M. Konrad (Max-Planck-Institut Göttingen, Germany). For the expression, purification and activity assay the described protocols were followed.[50,51]

The strains of *E. coli* BL21(DE3) harbouring the pT7–6[69] vector-based expression plasmids pSVW701 (*rmlB*), pSVW731 (*rmlC*) and pSVW711 (*rmlD*) containing the given genes for dTDP-L-rhamnose synthesis were constructed as follows: the individual genes were amplified by PCR from the genomic DNA of *Salmonella enterica*, serovar typhimurium (LT2), by the given primer pairs (*rmlB*: forward 5′-TATGTACGCAGTGCACTGGT-3′, reverse 5′-ATCACATCGGGAC-GAAGCTT-3′; *rmlC*: forward 5′-GGTTTATGCATGCTGATTGTGATTA-3′, reverse 5′-AAAAATCTTAATAGGATCCGACACG-3′; *rmlD*: forward 5′-GGGATCAGATTCGCGATTGG-3′, reverse 5′-AATGCCCTTACGCGTTTT-CA-3′). The amplified genes were cloned either blunt-end into the *Sma*I (*rmlB*, *rmlD*) cut or *Sph*I/*Bam*HI (*rmlC*) cut into the vector pUC18, which was cut the same way.[70] Gene sequences were monitored for correctness, and the genes were further cloned through *Eco*RI/*Bam*HI (*rmlB*, *rmlD*) or *Hin*dIII/*Bam*HI (*rmlC*) restrictions into the vector pT7–6, which was cut the same way. Overexpression of the RmlB, RmlC and RmlD proteins was identified and monitored in the crude extracts of *E. coli* BL21(DE3)/(pSVW701 or pSVW731 or pSVW711) by SDS-polyacrylamide gel electrophoresis[71] and under standard induction conditions yielded specific activities 479, 491 and 574 times higher, respectively, than the background activity of plasmid-free *E. coli* BL21(DE3), which expresses all three enzymes at low level. The production, purification and assays of the enzymes RmlB, RmlC and RmlD were performed as reported earlier.[41] A strain of *E. coli* BL21(DE3) harbouring the pPWW89 (pET16b-based) plasmid containing the *dnmU* gene from *Streptomyces peucetius* and yielding *N*-terminally His_6-tagged DnmU was constructed as described elsewhere.[57,72]

The *E. coli* strain BL21(DE3)pWW89 was grown aerobically in 5 L conical flasks with Luria–Bertani broth (2 L, 1% peptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (100 μg mL^{-1}), sorbitol (0.5 M) and betain (2.5 mM). The cultivation was carried out at 28 °C and with shaking (150 rpm) until the culture reached an $OD_{600nm} = 0.7$. After addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG, 0.5 mM) cells were cultivated for further 14 h at 28 °C. The cells were collected by centrifugation (Sorvall RC-SB, GS3 rotor) at 4000 rpm, washed three times with Tris/HCl (50 mM, pH 7.6) and stored at −20 °C.

A cell suspension (20%) in Tris-HCl buffer (50 mM, pH 7.6) containing NaCl (300 mM), imidazole (5 mM), $MgCl_2$ (1 mM), β-mercaptoethanol (ME, 10 mM) and BSA (0.1 mg mL^{-1}) was homogenized in a cell disintegrator (disintegrator S, IMA, Frankfurt, Germany) with glass beads (∅ = 0.3 mm) at 4 °C and 4000 rpm. The crude extract was obtained by centrifugation at 5000 rpm (Hettich Rotina 35R, Sigma) at 4 °C for 30 min. DnmU was purified by Immobilized Metal Ion Chromatography (IMAC) on a Ni^{2+}-NTA Superflow column (Qiagen, Hilden, 1.6 cm × 10.5 cm with 21 mL gel bed). The crude extract (67 mL, 469 mg protein), which was adjusted with ME (10 mM) and imidazole (20 mM), was loaded onto the column. After washing of the column with buffer A (50 mM Tris-HCl, pH 7.8, containing 300 mM NaCl, 20 mM imidazole, 10 mM ME) DnmU was eluted with imidazole (250 mM) in buffer A. The purity of DnmU was controlled by SDS-PAGE and Western blot analysis using anti-His_6 antibodies. A concentrated solution of DnmU (0.55 U mg^{-1} protein) was obtained by ultrafiltration (YM-10 ultrafiltration membrane, cut-off 10000 Da, Amicon, Witten, Germany) and stored at −20 °C until use for the synthesis of dTDP-deoxysugars. The activity of DnmU was determined as described for RmlC.[41,73]

Enzymatic synthesis of dTDP-α-D-glucose (2): The synthesis started from dTMP and sucrose with in situ regeneration of ATP by a combination of the enzymes dTMP-kinase, pyruvate kinase (PK) and SuSy1 in a repetitive batch mode with pH control. In a gently stirred Erlenmeyer flask, dTMP (**a**; 0.2 mmol, 73.2 mg), PEP (**b**; 0.3 mmol, 62.4 mg), ATP (**c**; 0.006 mmol, 3.3 mg), sucrose (**d**; 15 mmol, 5.13 g), dTMP-kinase (50 U), SuSy1 (100 U) and PK (1000 U) were dissolved in Tris/HCl buffer, (50 mM, pH 7.8, 50 mL) containing $MgCl_2$ (1 mM), KCl (1 mM), DTT (1 mM) and BSA (1 mg mL^{-1}; buffer B). This first batch was incubated at 30 °C for 17 h. After complete conversion of dTMP, by HPLC analysis, the product solution was separated from the enzymes by ultrafiltration to yield a residual volume of 10 mL. A second batch was started by the addition of fresh substrate solution (**a**–**d** in buffer B, 40 mL) and treated as described above. Because of the dilution the average concentration of the nine subsequent batches was 3.2 mM. Ten batches of dTMP (1.64 mmol) were added over a total incubation time of 150 h. The product solution (410 mL) contained **2** (1.54 mmol, 618 mg), which corresponds to a yield of 94% with reference to dTMP. The product solution was subjected to anion-exchange chromatography on Dowex 1 × 2, Cl^- form (2.6 × 34 cm) with a linear NaCl gradient (0–1 M) in distilled water (flow rate of 6 mL min^{-1}). The fractions containing **2** (HPLC analysis) were pooled, and nucleotides (dTMP, dTDP, ATP) were hydrolyzed by the addition of alkaline phosphatase. After ultrafiltration the product solution was concentrated by evaporation in vacuo to a volume of 40 mL. Desalting of the product was carried out at 4 °C on Sephadex G-10 (5.0 × 85 cm, 2 mL min^{-1}). The fractions analyzed by HPLC were pooled and stored at −20 °C. After lyophilization, **2** (1.02 mmol) was obtained as a white powder (618 mg, in an overall yield of 62% with reference to dTMP). HPLC analysis showed a purity of 99%. ^{1}H NMR (400 MHz, D_2O): $\delta = 7.66$ (s, 1H; H-6), 6.27 (t, $J = 7.1$ Hz, 1H; H-1′), 5.52 (dd, $J = 7.1$, 3.2 Hz, 1H; H-1″), 4.55 (m, 1H; H-3′), 4.11 (m, 3H; H-4′, CH_2-5′), 3.83 (m, $J = 13.3$, 3.8 Hz, 2H; H-6″), 3.76 (dt, $J = 9.4$, 3.2 Hz, 1H; H-2″), 3.70 (m, $J = 9.6$, 9.4 Hz, 1H; H-3″), 3.45 (m, $J = 9.6$, 3.8 Hz, 1H; H-5″), 3.38 (t, $J = 9.6$ Hz, 1H; H-4″), 2.28 (m, 2H; CH_2-2′), 1.85 ppm (s, 3H; CH_3); ^{13}C NMR (100 MHz, D_2O): $\delta = 166.8$ (s, C-4), 152.0 (s, C-2), 137.6 (s, C-6), 112.0 (s, C-5), 95.8 (d, C-1″), 85.5 (d, C-4′), 85.2 (s, C-1′), 73.0 (s, C-3″), 71.8 (d, C-2″), 71.7 (s, C-3′), 71.2 (s, C-4″), 69.4 (s, C-5″), 65.7 (d, C-5′), 60.5 (s, C-6″), 38.8 (s, C-2′), 11.9 ppm (s, C5-CH_3).

Enzymatic synthesis of dTDP-6-deoxy-4-keto-α-D-glucose (3): The synthesis started from dTMP and sucrose with in situ regeneration of ATP by a combination of the enzymes dTMP-kinase, pyruvate kinase (PK) and SuSy1 and RmlB in a repetitive batch mode under pH control. In a gently stirred Erlenmeyer flask, dTMP (**a**; 40 μmol, 14.7 mg), PEP (**b**; 60 μmol, 12.5 mg), ATP (**c**; 1.2 μmol, 0.66 mg), sucrose (**d**; 3 mmol, 1.0 g), dTMP-kinase (10 U), SuSy1 (30 U), RmlB (50 U) and PK (200 U) were dissolved in Tris/HCl buffer, (50 mM, pH 7.8, 10 mL), containing $MgCl_2$ (1 mM), KCl (1 mM), DTT (1 mM) and BSA (1 mg mL^{-1}; buffer B). This first batch was incubated at 30 °C for 5 h. After additional feeding of the substrates **a** and **b** and incubation for 7 h, the product solution from the first batch was separated from the enzymes by ultrafiltration to yield a residual volume of 1 mL. A second batch was started by the addition of 9 mL of fresh substrate solution (**a**–**d** in buffer B), feeding of **a** and **b**, and treated as described before. In the last batch, **a** and **b** were fed twice. dTMP (0.44 mmol) was added in five batches over a total incubation time of 77.5 h. The product solution (46 mL) contained **3** (0.43 mmol, 253 mg), with an average yield of 97% (with reference to dTMP). The product was isolated as described for **2**. After lyophilization, **3** was obtained as white powder (0.31 mmol, 183.5 mg, 72.1% overall yield with reference to dTMP). HPLC analysis showed a purity of 91%. ^{1}H NMR (400 MHz, D_2O): $\delta = 7.75$ (d, $J = 1.1$ Hz, 1H; H-6), 6.34 (t, $J = 7.0$ Hz, 1H; H-1′), 5.55 (dd, $J = 7.0$ and 3.6 Hz, 1H; H-1″), 4.62 (m, 1H; H-3′), 4.18 (m, 3H; H-4′ and CH_2-5′), 4.09 (q, $J = 6.6$ Hz, 1H; H-5″), 3.78 (d, $J = 10.0$ Hz,

1H; H-3″), 3.61 (ddd, J=10.0, 3.6 and 3.4 Hz, 1H; H-2″), 2.36 (m, 2H; CH_2-2′), 1.93 (s, 3H; CH_3), 1.22 ppm (d, J=6.6 Hz, 3H; H-6″); ^{13}C NMR (100 MHz, D_2O): δ=167.0 (s, C-4), 152.2 (s, C-2), 137.8 (s, C-6), 112.2 (s, C-5), 95.8 (s, C-1″), 94.1 (s, C-4″ hydrated), 85.8 (s, C-4′), 85.4 (s, C-1′), 73.6 (s, C-3″), 71.4 (s, C-3′), 71.0 (s, C-2″), 70.0 (s, C-5″), 65.9 (s, C-5′), 39.0 (s, C-2′), 12.1 (s, CH_3), 11.7 ppm (s, C-6″).

Enzymatic synthesis of dTDP-β-L-rhamnose (4): The synthesis started from **3** with in situ regeneration of NADH by a combination of the enzymes RmlC or DnmU, RmlD and FDH in a repetitive batch mode under pH control. In a gently stirred Erlenmeyer flask, **3** (**a**; 24 μmol, 14.2 mg), NAD^+ (**b**; 5 μmol, 3.6 mg), ammonium formate (**c**; 2 mmol, 126.1 mg), RmlC or DnmU (2 U), RmlD (2 U) and FDH (10 U) were dissolved in Tris/HCl buffer (50 mM, pH 7.8, 10 mL), containing $MgCl_2$ (1 mM), KCl (1 mM), DTT (1 mM) and BSA (1 mg mL^{-1}; buffer B). This first batch was incubated at 30 °C for 12 h. The product solution from the first batch was separated from the enzymes by ultrafiltration to yield a residual volume of 1 mL. A second batch was started by the addition of fresh substrate solution (**a**–**c** in buffer B, 9 mL) and treated as described above. Because of the dilution the average concentration of the nine subsequent batches was 2.24 mM. Compound **3** (67.2 μmol) was added in three batches over a total incubation time of 32 h. The product solution (28 mL) contained **4** (63.2 μmol, 37.4 mg) with an average yield of 94% (with reference to **3**). The product was isolated as described for **2**. Desalting of the product was carried out at 4 °C on Sephadex G-15 (2.5 cm×90 cm, 1 mL min^{-1}). After lyophilization, **4** was obtained as a white powder (24.1 μmol, 14.3 mg, 35.9% overall yield with reference to **3**). HPLC analysis showed a purity of 99%. ^{1}H NMR (400 MHz, D_2O): δ=7.73 (d, J=1.0 Hz, 1H; H-6), 6.34 (t, J=6.7 Hz, 1H; H-1′), 5.21 (brd, J=8.6 Hz, 1H; H-1″), 4.62 (m, 1H; H-3′), 4.18 (m, 3H; H-4′ and CH_2-5′), 4.08 (brd, J=3.3 Hz, 1H; H-2″), 3.64 (dd, J=9.5 and 3.3 Hz, 1H; H-3″), 3.44 (dq, J=9.5, 5.9 Hz, 1H; H-5″), 3.37 (t, J=9.5 Hz, 1H; H-4″), 2.36 (m, 2H; CH_2-2′), 1.92 (s, 3H; CH_3), 1.30 ppm (d, J=5.9 Hz, 3H; H-6″).

With DnmU a synthesis yield of 97% (65.2 μmol with reference to **3**) was achieved (32.2 μmol **4** after isolation, 19.1 mg, 47.9% overall yield with reference to **3**). ^{1}H NMR (400 MHz, D_2O): δ=7.73 (d, J=1.0 Hz, 1H; H-6), 6.34 (t, J=6.7 Hz, 1H; H-1′), 5.21 (brd, J=8.6 Hz, 1H; H-1″), 4.62 (m, 1H; H-3′), 4.18 (m, 3H; H-4′, CH_2-5′), 4.08 (brd, J=3.3 Hz, 1H; H-2″), 3.64 (dd, J=9.5, 3.3 Hz, 1H; H-3″), 3.44 (dq, J=9.5, 5.9 Hz, 1H; H-5″), 3.37 (t, J=9.5 Hz, 1H; H-4″), 2.36 (m, 2H; CH_2-2′), 1.92 (s, 3H; CH_3), 1.30 ppm (d, J=5.9 Hz, 3H; H-6″).

Synthesis of dTDP-α-D-quinovose (6) and dTDP-α-D-fucose (7): Purified **3** (12.5 μmol, 7.4 mg) was dissolved in distilled water (12.5 mL). Four aliquots of $NaBH_4$ (2.5 μmol each) were subsequently added at hourly intervals with monitoring of the reaction by HPLC. After complete conversion of **3** a yield of 91.2% was calculated for the mixture of epimers according to their peak areas. The peaks of both epimers could be assigned by comparison with the elution profile of the 4-epimers UDP-Glc/UDP-Gal, which are separated by the applied HPLC method. The ratio of the peak areas of **6** and **7** was 7:4. dTDP as decomposition product was hydrolyzed by alkaline phosphatase (3 h). After ultrafiltration the product solution was desalted on Sephadex G-15 as described above. After lyophilization, a mixture of **6** and **7** (10.8 μmol, 6.4 mg, 86.4% overall yield with reference to **3**) was obtained with a purity of 95% according to HPLC analysis. Main component **6**: ^{1}H NMR (400 MHz, D_2O): δ=7.74 (s, 1H; H-6), 6.34 (t, J=7.0 Hz, 1H; H-1′), 5.53 (dd, J=7.0, 3.6 Hz, 1H; H-1″), 4.62 (m, 1H; H-3′), 4.17 (m, 3H; H-4′, CH_2-5′), 3.97 (dq, J=9.5, 6.2 Hz, 1H; H-5″), 3.71 (t, J=9.5 Hz, 1H; H-3″), 3.52 (dt, J=9.5, 3.6 Hz, 1H; H-2″), 3.15 (t, J=9.5 Hz, 1H; H-4″), 2.36 (m, 2H; CH_2-2′), 1.93 (s, 3H; CH_3), 1.27 ppm (d, J=6.2 Hz, 3H; H-6″).

Minor component **7:** ^{1}H NMR (400 MHz, D_2O): δ=7.74 (s, 1H; H-6), 6.34 (t, J=7.0 Hz, 1H; H-1′), 5.56 (dd, J=6.8 and 3.6 Hz, 1H; H-1″), 4.62 (m, 1H; H-3′), 4.28 (brq, J=6.6 Hz, 1H; H-5″), 4.17 (m, 3H; H-4′ and CH_2-5′), 3.91 (dd, J=10.5 and 3.2 Hz, 1H; H-3″), 3.81 (brd, J=3.2 Hz, 1H; H-4″), 3.74 (dt, J=10.5 and 3.2 Hz, 1H; H-2″), 2.36 (m, 2H; CH_2-2′), 1.93 (s, 3H; CH_3), 1.21 ppm (d, J=6.6 Hz, 3H; H-6″).

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