Application of the Nucleotidylyltransferase E_p toward the Chemoenzymatic Synthesis of dTDP-Desosamine Analogues**

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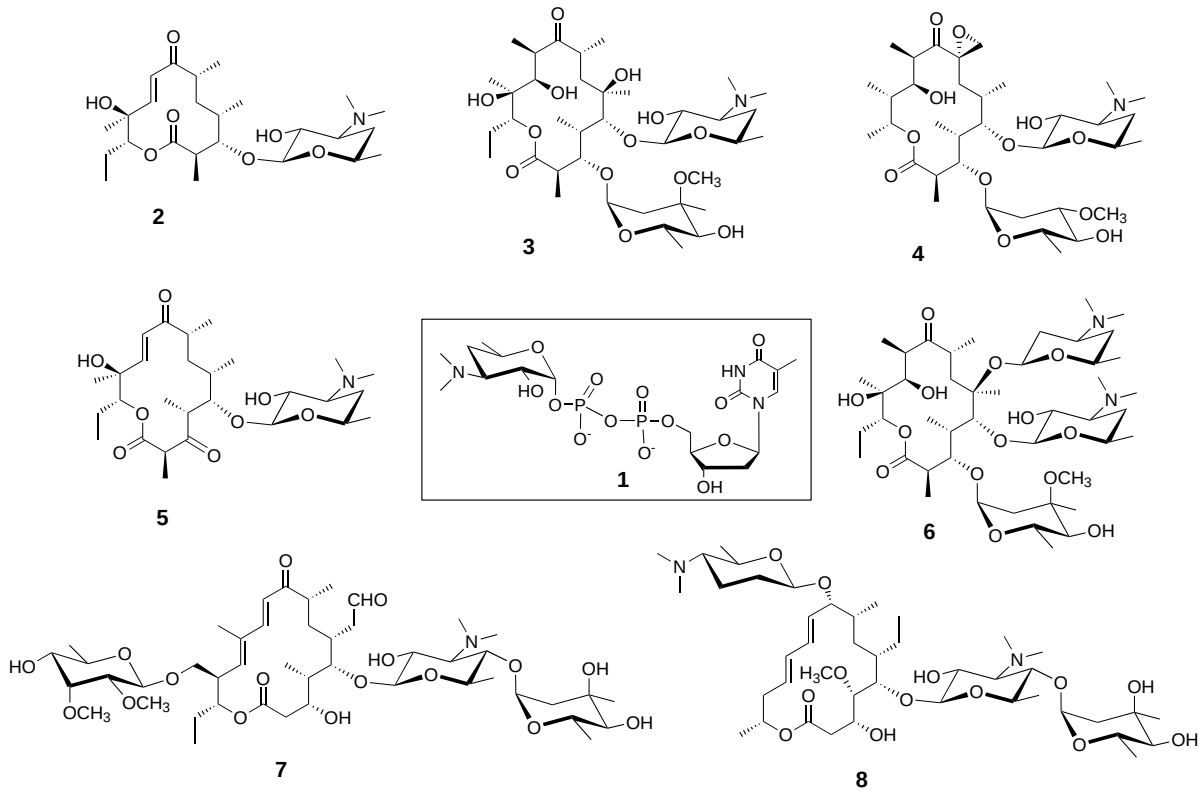
KEYWORDS:

antibiotics · enzyme catalysis · glycorandomization · glycosyltransferases · nucleotides

One of the most notable macrolide sugar substituents, shared by vastly different macrolide family members such as methymycin (**2**, Scheme 1), erythromycin (**3**), oleandromycin (**4**), pikromycin (**5**), and megalomicin (**6**), is β-D-desosamine (3,3-*N*,*N*-dimethylamino-3,4,6-trideoxy-β-D-xylo-hexopyranose).[1] Close analogues of this important sugar are also found as appendages of other unique macrolides including tylosin (**7**) and spiramycin (**8**). Structure – activity studies support the important contribution of this glycoside to macrolide antibacterial activity,[2] metabolism,[3] and tissue distribution,[4] and it has been suggested that methods to alter this sugar may lead to macrolide derivatives with enhanced properties. Elegant biosynthetic studies reveal that the attachment of desosamine requires eight enzymes (DesI – DesVIII) and culminates in the glycosyl transfer event from the activated pyrimidine nucleoside diphosphosugar dTDP-α-D-desosamine (**1**, Scheme 1; dTDP = deoxythymidine diphosphate).[5] Yet the synthetic inaccessibility of dTDP-desosamine and corresponding biosynthetic intermediates has hampered in vitro biosynthetic studies and attempts to exploit the critical desosaminyltransferase for macrolide in vitro 'glycorandomization'. To date, the best reported dTDP-desosamine synthesis requires 16 chemical transformations, an enzymatic resolution, and a final enzymatic *N*,*N*-dimethylation with an overall yield of ≈4% (assuming 100% conversion for both enzymatic steps).[6]



Scheme 1. *Natural products bearing desosamine and desosamine-related glycosides. The natural activated precursor for enzymatic desosamine attachment (dTDP-desosamine) is shown in the box.*

Salmonella E_p is an enzyme that converts α-glucose-1-phosphate (Glc-1-P) and deoxythymidinetriphosphate (dTTP) into dTDP-α-D-glucose and is unique in that it displays notable indifference toward other sugar phosphate substrates; it thereby presents the opportunity to efficiently generate nucleoside diphosphosugar libraries.[7] To further our efforts in expanding

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[**] *dTDP = deoxythymidine diphosphate.*

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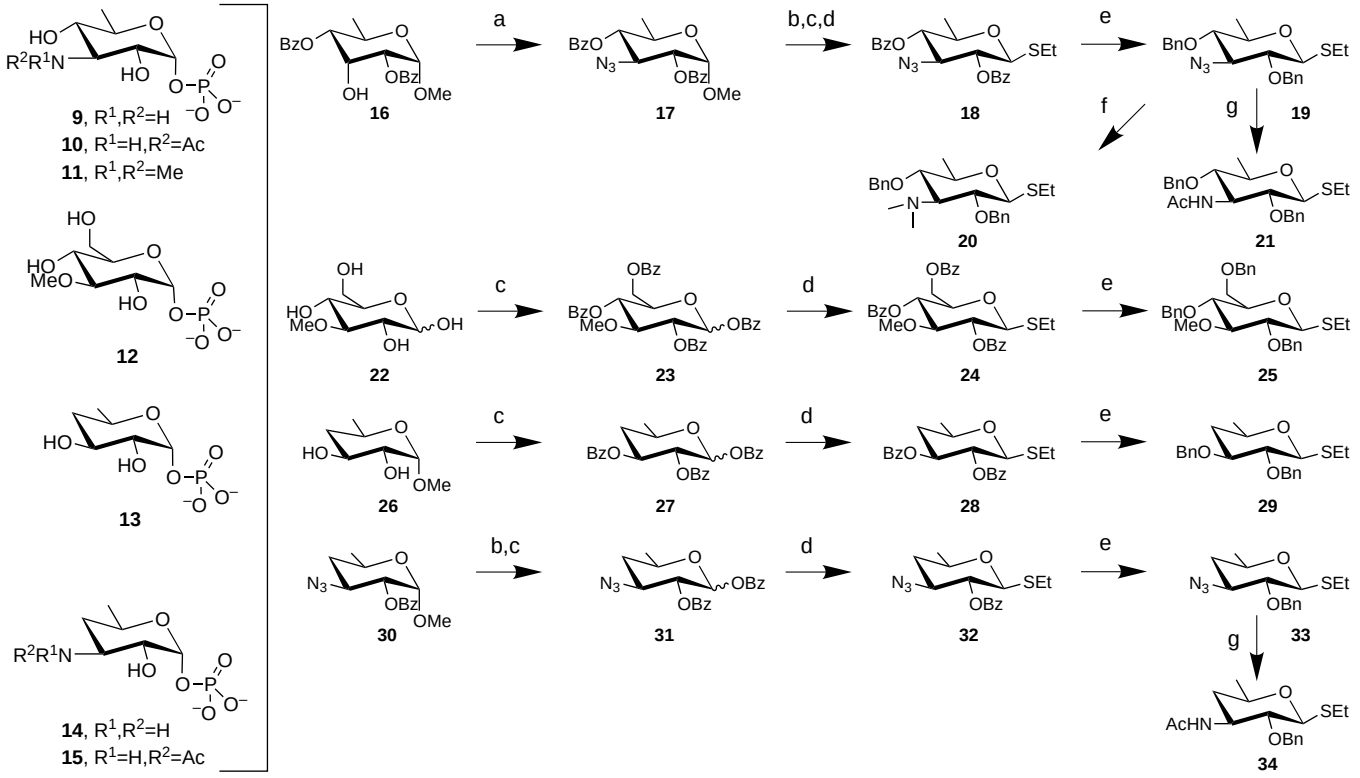


Scheme 2. *An overview of the key steps in the syntheses of substrate analogues for E_p. Reaction Conditions: a) 1) Tf_2O, pyr, 2) NaN_3 (88% average overall yield, two steps); b) 1) MeONa, 2) H_2SO_4 (53% average yield, two steps); c) BzCl, pyr (90% average yield); d) TMSSEt, ZnI_2 (82% overall yield); e) 1) MeONa, 2) NaH, BnBr (89% average overall yield, two steps); f) 1) $SnCl_2$, PhSH, Et_3N, 2) H_2CO, $NaBH_3CN$, AcOH (86% average yield, two steps); g) 1) $SnCl_2$, PhSH, Et_3N, 2) Ac_2O, pyr (82% average overall yield, two steps); final steps (not shown): 1) phosphorylation, 2) reductive deprotection, 3) cation exchange to give the Na^+ salt (50% average overall yield, three steps). Tf = triflate = trifluoromethanesulfonyl, pyr = pyridine, TMS = trimethylsilyl, Bz = benzoyl, Bn = benzyl.*

the general synthetic utility of nucleotidylyltransferases, we report herein the application of E_p,[8] and specific designed E_p mutants,[9] toward the synthesis of dTDP-desosamine analogues. The significance of this work stems from the ability to efficiently generate analogues applicable to the development of in vitro glycorandomization systems[1, 8–10] and the mechanistic study of unique deoxysugar biosynthetic pathways. In addition, this work provides further fundamental information regarding the substrate specificity of this important class of enzyme.

None of the compounds tested as substrates for E_p in this study (**9** – **15**) were commercially available. In all cases, precursors were activated through the formation of 1-ethylthio-β-D-pyranosides (**19** – **21**, **25**, **29**, **33**, **34**) and phosphorylated by the reaction with dibenzyl phosphate as previously described.[8] The 3-amino-3,6-dideoxy series (**9** – **11**) were derived from methyl (*R*)-4,6-*O*-benzylidine-α-D-glucopyranoside[11] by C-6 deoxygenation, C-3 inversion (standard pyridinium chlorochromate (PCC) oxidation followed by $NaBH_4$ reduction), subsequent C-3 hydroxy group activation (as the triflate), and displacement by sodium azide; this provided **17**. Standard transformations led to intermediate **19** from which the syntheses of **9**, **10**, and **11** diverged. The amino- and acetamido-substituted **9** and **10** followed previously reported strategies from **19** and **21**, respectively.[8b, 12] The reduction of **19** and subsequent exhaustive reductive methylation[12] led to the dimethyl-substituted precursor **20**. Substrate **12** was derived from commercially available **22** following similar activation and phosphorylation strategies,[8] while substrate **13** derived from the 4,6-dideoxy glucoside **26**,[13a] generated by C-4/C-6 activation and reductive deoxygenation in a fashion similar to a previously described strategies.[8a] Finally, substrates **14** and **15** were derived from the 3-azido-3,4,6-trideoxy glucoside **30**,[13b] by generation of the 3-amino-3,4,6-trideoxy precursor **33** and the 3-acetamido-3,4,6-trideoxy precursor **34**, in a manner similar to previously described strategies.[8b]

To evaluate the utility of E_p toward the generation of dTDP-desosamine derivatives, E_p, hexopyranosyl phosphate substrate (**9** – **15**), TTP, and inorganic pyrophosphatase were incubated at 37 °C for 30 min and the extent of product formation determined by HPLC.[14] For each assay, confirmation of the product was based upon LC – MS and, in some cases, coelution with available standards. In addition to testing wild-type enzyme in these assays, mutants known to have enhanced promiscuity (L89T, T201A, Y117F, and W224H)[9] were also examined. Control

reactions in the absence of E_p, hexopyranosyl phosphate, Mg^{+2}, or TTP showed no product formation.

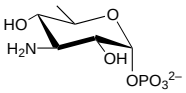
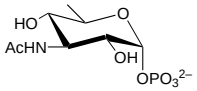
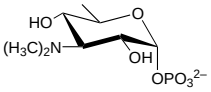
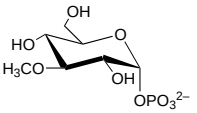
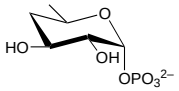
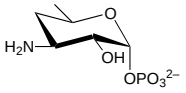
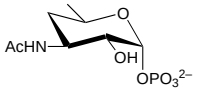
The fundamental goal of this study was to assess the utility of E_p in simplifying the synthesis of nucleotide sugar pools particularly related to dTDP-desosamine. Table 1 clearly illustrates that E_p is advantageous for this task. Of the seven unnatural substrates tested (**9** – **15**), four provide appreciable amounts of product (> 75 %) under these conditions. A comparison among the 3,6-dideoxy derivatives (**9** – **11**) reveals that C-3 amino and acetamido substitution suprisingly has no effect upon activity while the *N*,*N*-dimethyl substitution at this position completely abolishes activity. This is consistent with the previously demonstrated ability of this enzyme to tolerate 6-deoxy-, 3-amino-3-deoxy-, and 3-acetamido-3-deoxy-α-D-glucopyranosyl phosphates as substrates.[8]

The lack of activity in the presence of **11** is likely to be a result of sterics and/or hydrogen-bond character/charge constraints. The pK_a value of the C-3 tertiary amine group in **11** is ≈ 10,[15a] of the C-3 primary amine in **9** is ≈ 9,[15a] and of the C-3 amide in **10** is ≈ 0.4,[14b] a fact suggesting that **9** and **11** may primarily exist in a protonated species under typical assay conditions (pH 7.5 – 8.0). An attempt to alleviate this possibility, by assays at higher pH values, failed to enhance activity with **11**.[16] In the natural substrate (Glc-1-P), there are clear hydrogen-bond contacts between the C-3 hydroxy group and the side chains of Glu162, Thr201, and the backbone amide of Gly147.[9] Previous mutagenesis experiments revealed slight yield enhancements with bulky C-2 substitutions in a Thr201Ala mutant but Glu162 mutations had little effect on substrate specificity.[9a, 17] While hydrogen-bonding character clearly differs among this current substrate series, modulation of the ability to donate a C-3 hydrogen bond (for example, **12**, 75 % conversion) does not significantly alter product yield. Thus, we conclude steric constraints may be the major contributor to the lack of product formation with **11**.

Interestingly, removal of the C-4 hydroxy group from members of this series (**14** and **15**), has a significant effect on activity. The average observed yield for substrate **14** is low (10.0 %) and no turnover is observed in the presence of **15**. Yet, in the absence of substitutions at C-3, removal of the C-4 hydroxy moiety (**13**, quantitative conversion) has no effect on activity. The Gly147 backbone amide makes hydrogen-bond contacts with both the substrate C-3 and C-4 hydroxyl groups and is the only observed C-4 contact point.[9] Based upon this, one might speculate that minimally a Gly147 to C-3 XH *or* C-4 XH hydrogen bond must be sustained to conserve catalytic integrity. As one begins to substantially perturb both contacts, such as in **15**, E_p is no longer able to properly bind and/or process the substrate.

In regard to the chemoenzymatic synthesis of dTDP-desosamine: although the conversion of **14** by E_p is low, the overall synthetic yield of substrate **14** from 2-*O*-benzoyl-4,6-dideoxy-α-D-erythro-hexopyranoside[13b] is 10.0 %. Thus, optimization of this method, in conjunction with the final DesVI-catalyzed methylation step described by Chang and Liu,[6] may prove the more efficient route to the challenging dTDP-desosamine target. More importantly, the presented methodology provides the ability to generate analogues of this important metabolic precusor, thus paving the way for potential structure – activity relationships among various bioactive secondary metabolites and/or the corresponding enzymes involved in constructing these compounds. In addition, this work provides an additional framework to support our ability to predict the synthetic utility and limitations of this unique nucleotidylyltransferase.

Table 1. *E_p-Catalyzed conversion of substrates.*

Substrate	TTP conversion [%][a]	Retention time [min][b]
9 	99[c]	10.8
10 	99[d]	24.1
11 	–[e]	–[e]
12 	75[c]	26.6
13 	99[c]	29.7
14 	10[c]	12.3
15 	–[e]	–[e]

[a] Percentage conversion = [AP/(AP + AT)] × 100, where AP = the product peak integration and AT represents the TTP peak integration. [b] Standard retention times: thymidinemonophosphate (TMP) 9.7 min; TDP 27.9 min; TTP 42.8 min; TDP-Glc 26.0 min. [c] Conversion with wild-type E_p. [d] Conversion with E_p T201A mutant. [e] No products observed.

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[14] Assays were conducted in a total volume of 50 μL with slight modification of the previously reported method:[8] 10 mM α-D-hexose-1-phosphate, 5 mM TTP, 50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (pH 8), 10 mM $MgCl_2$, 1 μg mL^{-1} bovine serum albumin, 5 U E_p, 4 U inorganic pyrophosphatase. Reactions were conducted at 37 °C for up to 30 min and the proteins were subsequently removed by boiling for 1 min and centrifugation at 10,000 × *g* for 30 min. The formation of new products was monitored by HPLC (Varian) analysis with UV detection at 254 nm. A phosphate buffer (30 mM potassium phosphate (pH 6.0), 5 mM tetrabutylammonium hydrogensulfate, 4% acetonitrile) and acetonitrile (0 to 50% acetonitrile over 50 min) were used as the mobile phase in combination with a reversed-phase column (Luna C18, 5 μm, 250 × 4.6 mm (Phenomenex)). Each putative substrate was tested with all available E_p variants and the best conversions (average of three independent assays) are illustrated in Table 1.
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[16] Assays at higher pH values were carried out as described (ref. [14]) but with different buffer conditions: 50 mM Tris/HCl, (pH 7, 7.5, 8.5) and 50 mM Tricine/NaOH (pH 9, 9.5, 10, 10.5). The corresponding yield with Glc-1-P at pH 10.5 was > 60% of that observed at pH 8.0.
[17] W. A. Barton, J. B. Biggins, J. Jiang, D. B. Nikolov, J. S. Thorson, unpublished data.

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