Glycosylations

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Substrate Specificity of the Macrolide-Glycosylating Enzyme Pair DesVII/DesVIII: Opportunities, Limitations, and Mechanistic Hypotheses**

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Macrolide antibiotics consist of a polyketide macrolactone with one or more deoxysugar residues attached. Macrolides target protein translation and are highly effective against gram-positive bacteria and represent an important class of compounds in our current antibiotic arsenal to treat pathogenic bacteria.[1] However, as with most broadly prescribed antibiotics, the rapid emergence of macrolide-resistant organisms dictates the continuous need for new macrolide analogues to help sustain our battery of effective agents against the constant onslaught of potentially lethal microbes.[2] The recent determination of the 3D structure of the 50S ribosomal subunit complexed with a variety of macrolides clearly reveals that the sugar substituents of these macrolides contribute to

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binding and sterically infringe upon the polypeptide exit tunnel—thereby preventing peptide-chain elongation.[3] Structural studies also show that the C5 disaccharide moieties of 16-membered macrolides, such as spiramycin and carbomycin, extend further into the exit tunnel toward the adjacent peptidyl transferase center. Moreover, a direct correlation between the length of truncated peptides released by a macrolide-bound ribosome and the length of the saccharide side chain of the macrolide has been established. Cumulatively, these studies clearly demonstrate the essential nature of interactions between rRNA and deoxy sugars of macrolide antibiotics and suggest that alterations of these appended carbohydrates may modulate binding and/or potentially circumvent certain resistance mechanisms.

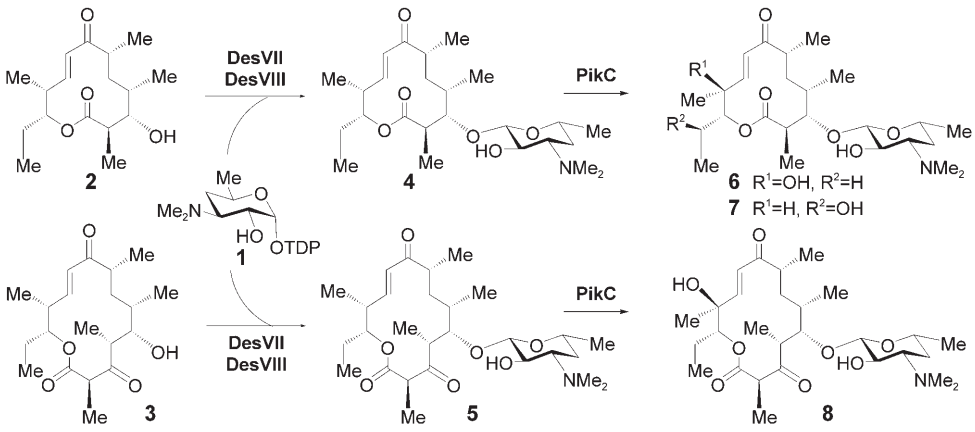
Given the architectural and synthetic complexity of macrolides, pathway engineering has emerged as an attractive means for in vivo analogue production, presenting new derivatives with variant aglycones and/or sugars.[4] These in vivo studies have also revealed a relaxed specificity of the responsible macrolide glycosyltransferases toward both their sugar and aglycone substrates.[5] In 2004 we successfully demonstrated the first in vitro activity of a purified macrolide glycosyltransferase, DesVII,[6] which catalyzes the attachment of TDP-desosamine (**1**) to 10-deoxymethynolide (**2**) or narbonolide (**3**) in the biosynthesis of methymycin (**6**), neomethymycin (**7**), pikromycin (**8**), and narbomycin (**5**) in *Streptomyces venezuelae* (Scheme 1).

This in vitro study revealed an unusual requirement for DesVII activity: the need for an additional protein partner, DesVIII. Recently, a similar requirement for an auxiliary protein (EryCII) was also established for EryCIII, which catalyzes the attachment of desosamine (**1**) to the related erythronolide core.[7] Interestingly, among the growing number of in vitro studies of related glycosyltransferases—including those for nonribosomal peptides,[8] aminocoumarins,[9] enediynes,[10] macrolactams,[11] macrolides,[6,7,12] and aromatic polyketides[13]—only five, including DesVII and EryCIII, have been shown to depend on a DesVIII homologue.[14] Although their function remains unclear, the genes coding for DesVIII homologues are only found within biosynthetic loci encoding for aminosugar-bearing metabolite biosynthesis where they typically reside directly upstream to the aminosugar glycosyltransferase gene.[15] Thus, DesVIII was initially implicated as necessary for the incorporation of aminosugar moieties in these metabolites.[6]

In study reported herein, we extend the established in vitro DesVII/DesVIII assay to assess the level of aglycone and sugar substrate tolerance by this catalytic pair. In addition to providing a set of new macrolides, this work reveals a notably stringent 6-deoxysugar requirement for DesVII/DesVIII-catalyzed glycosylation. More importantly, this work also reveals that the DesVIII "boost" is not limited to aminosugars and thus vastly contrasts the earlier "aminosugar carrier" hypothesis.

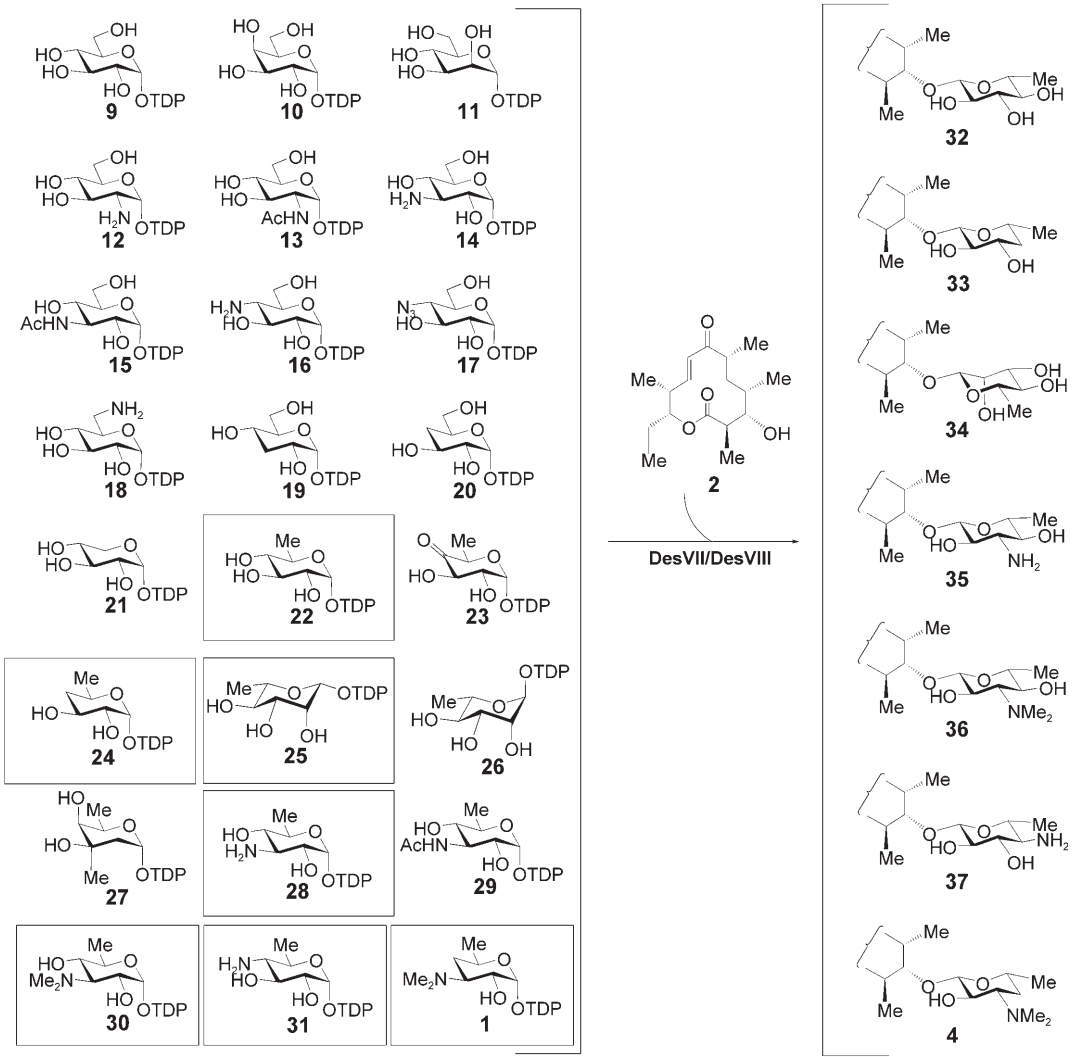
The recombinant DesVII protein was overproduced in *E. coli* as a C-terminal His_6-tagged fusion protein and purified as previously described.[6] The DesVIII protein was produced in *E. coli* as a fusion with maltose-binding protein at its N terminus. The fusion protein was cleaved by treatment with protease and untagged DesVIII protein was used in the assays together with DesVII.[16] A number of TDP-sugars were tested as donor substrates for DesVII/DesVIII with the native macrolide aglycone **2** (Scheme 2) as the acceptor. These include TDP derivatives of glucose **9**, galactose **10**, mannose **11**, and a series of amino and acetamido hexoses **12**–**18**. TDP-sugars deoxygenated at C3 and C4, **19** and **20**, as well as TDP-xylose **21** were also examined. As the natural substrate desosamine (**1**) is a 6-deoxyhexose, a variety of 6-deoxy sugars **22**–**27** and 6-deoxyaminosugars **28**–**31** were also tested.

Of the sugar nucleotides employed, compound **9** was commercially available, compounds **1**,[17] **26**,[18] and **28**[17b] were previously synthesized chemically, compound **30** was prepared by a chemoenzymatic method,[17b] and compounds **23**, **25**,[16] and **27**[19] were obtained through enzymatic synthesis. The remaining 16 TDP-sugars **10**–**22**, **24**, **29**, **31** were generated in situ through an engineered nucleotidylyltransferase (E_p) catalyzed chemoenzymatic route from the respective sugar-1-phosphates as previously described.[20] For each assay, TDP-sugar formation in situ was assessed by HPLC, the reaction mixture subsequently adjusted to pH 9, and DesVII, DesVIII, and **2** added. The progress of the glycosylation reaction was monitored by TLC. The products from samples showing positive TLC results were subsequently isolated by preparative TLC or HPLC, and their identities established by high-resolution MS and/or NMR spectroscopic analysis.[16]



Scheme 1. Final steps of the biosynthesis of macrolide antibiotics methymycin (**6**), neomethymycin (**7**), narbomycin (**5**), and pikromycin (**8**) in *Streptomyces venezuelae*. PikC = cytochrome P450 hydroxylase; TDP = thymidine diphosphate.

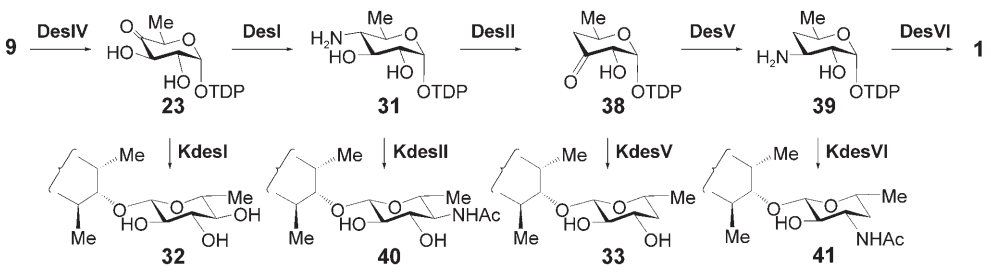
Of the 23 activated sugars tested, only seven were accepted by DesVII/DesVIII to give products **4** and **32**–**37** (Scheme 2), of which **35**–**37** have never been reported. The in vitro production of macrolides **32** and **33**, previ-



Scheme 2. Study of sugar nucleotide substrate specificity of DesVII/DesVIII using 10-deoxymethynolide (**2**) as acceptor.

ously identified shunt metabolites from *S. venezuelae* disruption mutants, suggests that reduction of the keto group of NDP-sugars **23** or **38** likely occurs prior to glycosylation (Scheme 3) in these engineered mutant strains.[5d,b] This order of events is further supported by the observation that **23** is not a substrate of DesVII/DesVIII.

Consistent with the early in vivo studies, a strict requirement for 6-deoxysugars was also observed in vitro. TDP-sugars bearing functional groups at C6 (e.g. **9**–**20**) or lacking C6 (e.g. **21**) failed as DesVII/DesVIII substrates, whereas fairly broad structural variation was tolerated among the 6-deoxyhexose substrates tested, including both D (e.g. **22, 24**) and L sugars (**25**). Aminosugars and *N*-alkyl aminosugars were found to be substrates (e.g., **1, 28, 30**, and **31**), but *N*-acyl aminosugars were not. The fact that aminosugar **28** is a substrate whereas *N*-acetamido sugar **29** is not supports our previous hypothesis that *N*-acetylation occurs after glycosylation in the production of **40** and **41** (Scheme 3).[5c,f,21]



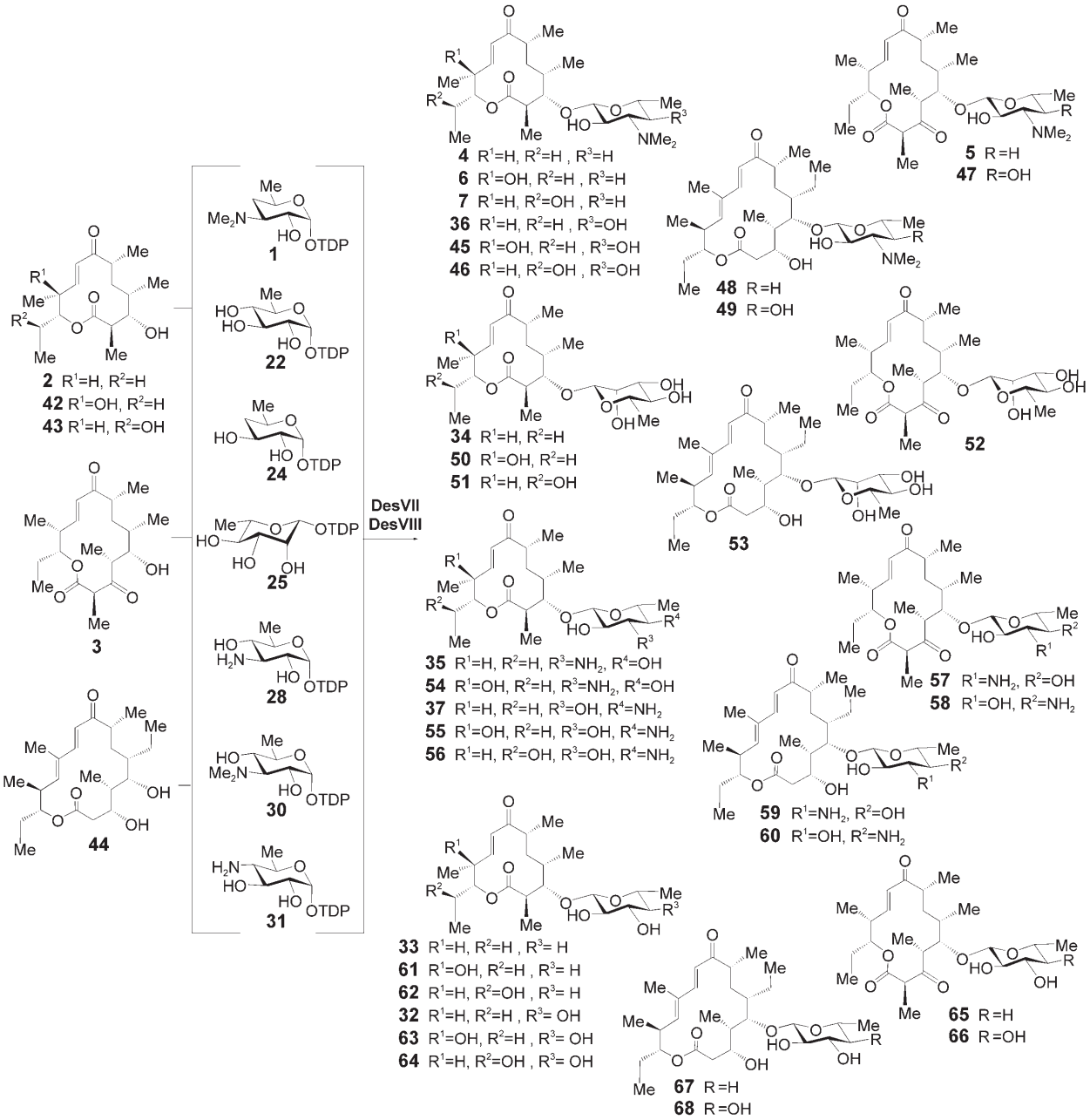
Scheme 3. Biosynthetic pathway to TDP-desosamine (**1**) from TDP-glucose (**9**). Also shown are macrolide products isolated from the fermentation broths of various gene-disruption mutants of *S. venezuelae*. Kdes = *S. venezuelae* mutants in which the respective des genes are inactivated.

Furthermore, C3 alkylation and/or C2 deoxygenation appear to be detrimental (e.g. **27**) whereas the correct anomeric configuration (e.g. **25** versus **26**) is essential for DesVII/DesVIII activity. Most importantly, parallel assays in the absence of DesVIII led to a dramatic decrease in DesVII activity (ranging from a

more than 30-fold decrease in yield to no product at all), including those assays with substrates that lack amino functionality (e.g. **24** and **25**).[16] Clearly, the DesVIII augmentation effect is not limited to aminosugar substrates only.

Given the well-characterized in vivo aglycone tolerance of DesVII/DesVIII,[22] we next set out to explore this potential in vitro, particularly in the context of non-natural sugar donors. Consistent with earlier observations, we found DesVII/DesVIII to be capable of adding desosamine to the natural acceptors 10-deoxymethynolide (**2**) and narbonolide (**3**) to give **4** and **5**, respectively. Interestingly, the hydroxylated analogues of **2**, methynolide (**42**) and neomethynolide (**43**), can also be glycosylated to yield **6** and **7**, respectively. Remarkably, all six confirmed non-natural in vitro sugar substrates **22, 24, 25, 28, 30**, and **31** from the studies described above, were also successful DesVII/DesVIII substrates with aglycones **2, 3, 42**, and **43** (Scheme 4). In the context of the previous hypothesis that PikC-catalyzed aglycone hydroxylation (i.e. the conversions of **4** into **6**/**7** and **5** into **8**) is a postglycosylation event,[23] these results demonstrate a tolerance of DesVII/DesVIII towards the hydroxylation pattern of the aglycone and imply some flexibility in the glycosylation/hydroxylation sequence of biosynthetic steps. Many of the macrolides first identified through pathway engineering (**32**,[5d] **34, 50–52**,[5e] **45–46**,[14a] **61–62**,[5b] **63–66**[24]) were also identified in this study.

Interestingly, we found **1, 22, 24, 25, 28, 30**, and **31** to be substrates with the 16-membered tylactone (**44**) as well to give **48, 68, 67, 53, 59, 49**, and **60**, respectively (Scheme 4). Of these products, **48** and **49** had been previously identified in vi-



Scheme 4. Structures of macrolides identified in this study.

vo,[25,14a] while others have not been reported. Also, although **44** contains two potential glycosylation sites, the DesVII/DesVIII system was regioselective for C5. Most importantly, parallel assays lacking DesVIII again led to a dramatic reduction in the yield of the glycosylated products. Such an ability to utilize a non-natural aglycone and a non-natural sugar in the coupling reaction is unusual for enzyme catalysis, but appears to be more common among glycosyltransferases involved in the biosynthesis of secondary metabolites.[4,5] The demonstrated aglycone as well as sugar promiscuity of this system presents an opportunity for macrolide glycodiversification.

In summary, the in vitro DesVII/DesVIII characterization described herein not only demonstrates its potential for macrolactone glycodiversification,[26] but also further expands our understanding of the DesVII/DesVIII glycosylation machinery. Based on these studies, DesVII/DesVIII exhibits a stringent 6-deoxysugar requirement but an otherwise relaxed substrate specificity toward both TDP-sugar donor and aglycone acceptor. The catalytic capabilities of DesVII/DesVIII, as indicated by the formation of 19 previously unreported macrolides in this limited exploration, hold high promise in biosynthetic application to construct new macrolide derivatives. More importantly, these results reveal that DesVIII equally augments DesVII activity toward both aminosugars and non-aminosugars. The lack of preferential aminosugar enhancement contrasts the previously proposed "aminosugar-carrier" role for this intriguing protein. DesVIII has also been speculated to play a role in the enhancement of the aglycone–glycosyltransferase interaction considering the high hydrophobic nature of the aglycones **2** and **3**. However, given the fact that the DesVIII augmentation effect holds for both hydrophobic (such as **2** and **3**) and more-hydrophilic aglycones (such as **42** and **43**), a specific "aglycone-carrier" role for DesVIII may also be called into question. Since DesVII shows good sequence homology to members of the glycosyltransferase GT-B superfamily, which contain two distinct domains for harboring the aglycone and TDP-sugar substrate, it is more likely that binding of DesVIII to DesVII may optimize a DesVII conformational change bringing these two domains to close proximity during coupling, or may simply facilitate the proper folding of DesVII and thereby improve its catalytic efficiency. In fact, our preliminary experiments showed that DesVIII is only required for initial activation of DesVII and the activated DesVII can catalyze the glycosyl transfer alone. Interestingly, a similar observation was recently reported for activation of glycosyltransferase EryCIII by DesVIII homologues EryCII and AknT.[7] The authors proposed that auxiliary proteins induce a one-time conformational change of glycosyltransferase to its active form. Experiments are in progress to investigate further the catalytic properties of DesVII and DesVIII.

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