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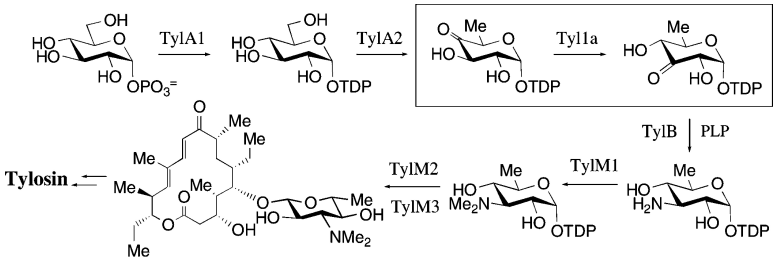

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TDP-Mycaminose Biosynthetic Pathway Revised and Conversion of Desosamine Pathway to Mycaminose Pathway with One Gene

Charles E. Melançon III, Wei-luen Yu, and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy and Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712

Received June 10, 2005; E-mail: h.w.liu@mail.utexas.edu

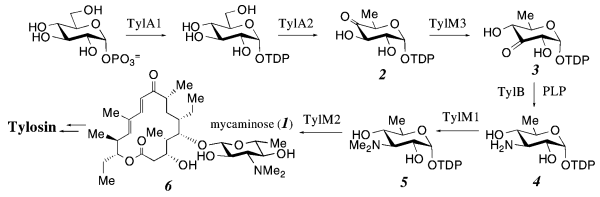
Deoxyaminosugars, such as D-mycaminose (**1**, Scheme 1), are present in many biologically active secondary metabolites and are frequently critical for the bioactivity of the parent compounds.[1] As part of our effort to reengineer secondary metabolite sugar biosynthetic machinery for glycosylation of various aglycones,[1b] we investigated the biosynthesis of D-mycaminose in *Streptomyces fradiae*, the producer of the mycaminose-containing macrolide antibiotic tylosin. The entire tylosin biosynthetic gene cluster has previously been identified and sequenced,[2] and the genes *tylM3*, *tylM2*, *tylM1*, and *tylB* were proposed to encode proteins involved in mycaminose production and/or attachment.[3]

The functions of the encoded proteins were assigned based on sequence comparison with known genes in the data bank. Accordingly, it was proposed that TylB catalyzes the formation of TDP-3-amino-3,6-dideoxy-D-glucose (**4**) from the corresponding keto sugar **3**, TylM1 converts **4** to TDP-D-mycaminose (**5**), and TylM2 functions as the mycaminosyl transferase whose product is 5-*O*-mycaminosyl−tylactone (**6**) (Scheme 1). TylM3, which has low sequence identity (∼20%) to P450 enzymes and lacks the conserved active site cysteine and heme binding motif, was proposed to be an isomerase catalyzing the conversion of **2**, a common intermediate for the biosynthesis of 6-deoxyhexoses, to **3**.[3b] The proposed roles of TylB and TylM1 were subsequently confirmed by functional analysis, as shown in Scheme 1.[3b,4]

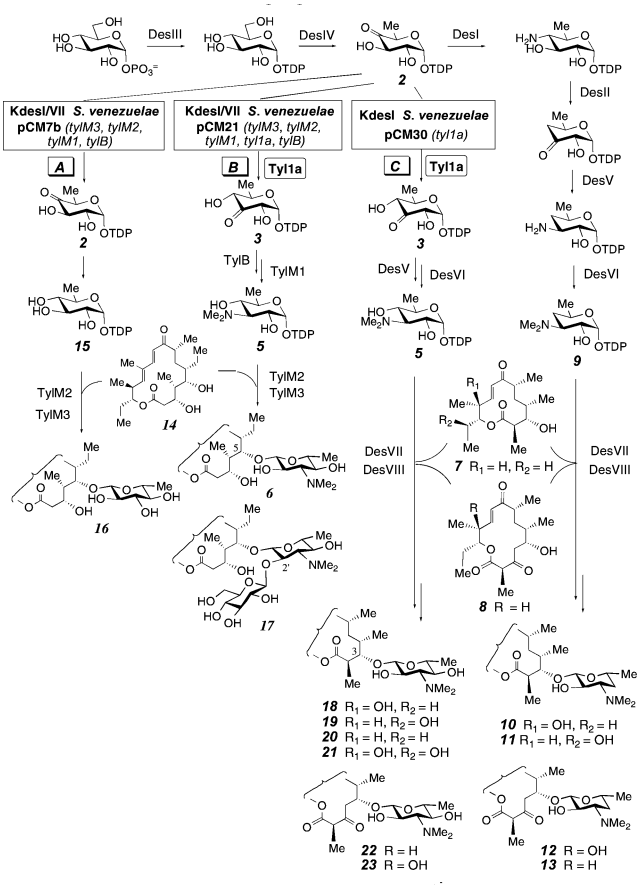
To verify the functions of the remaining genes in the mycaminose pathway, efforts were made to overexpress and purify TylM2 and TylM3. However, low expression and poor solubility of these proteins in *E. coli* hampered these efforts. Thus, we opted to employ an in vivo expression strategy to reconstitute the entire mycaminose pathway in a heterologous host. Validation of the putative functions of TylM2 and TylM3 would be made by the observation of mycaminose production. The strain *Streptomyces venezuelae*, which produces methymycin (**10**), neomethymycin (**11**), pikromycin (**12**), and narbomycin (**13**), was chosen as the host. These macrolide antibiotics contain D-desosamine as the sole sugar component attached to a macrolactone: 10-deoxymethynolide (**7**) for **10**/**11** and narbonolide (**8**) for **12**/**13**, respectively. The biosynthesis of **10**−**13**, especially the formation of TDP-desosamine (**9**) (Scheme 2, genes *desI*−*desVIII*), has been investigated in detail.[4a,5]

The previously constructed KdesI mutant[5c] of *S. venezuelae*, where the *desI* gene has been disrupted, was used as the template for making a KdesI/KdesVII mutant, which would serve as the host for heterologous expression of the mycaminose biosynthetic genes.[6] With both *desI* and *desVII* gene disrupted in the mutant, the desosamine pathway would stop at **2**, and no glycosylated product is expected due to the absence of glycosyltransferase in the double mutant. A pAX617[6]-derived expression plasmid, pCM7b, carrying *tylM3*, *tylM2*, *tylM1*, and *tylB* was constructed and subsequently introduced into the KdesI/VII mutant. A small-scale culture of the resulting mutant strain, KdesI/VII-pCM7b, was grown in antibiotic

Scheme 1



Scheme 2



production media[7] supplemented with tylactone (**14**). It was predicted that **2** would be converted to TDP-mycaminose (**5**) by the heterologously expressed TylM3, TylB, and TylM1, and then attached by TylM2 to the exogenously introduced **14**. Indeed, TLC analysis of the chloroform extracts of the culture broth of this mutant exhibited a new polar spot. However, high-resolution MS analysis indicated that this new compound is most likely quinovosyl−tylactone (**16**, calcd for $C_{29}H_{48}O_9$ $(M + 1)^+$ 541.3377, found 541.3379), and not the anticipated mycaminosyl−tylactone (**6**) (Scheme 2, route A).

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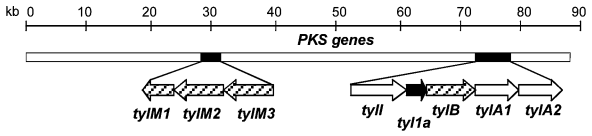

Figure 1. The tylosin gene cluster highlighting the positions of *tyl1a* and the mycaminose biosynthetic genes.

It should be noted that a quinovosyl derivative of 10-deoxymethynolide was found to be the major glycosylated product made by the original KdesI mutant of *S. venezuelae*, in which TDP-quinovose (**15**) is believed to be derived from **2** via C-4 reduction by an endogenous reductase.[5c] The fact the KdesI/VII-pCM7b mutant produced **16** containing quinovose instead of mycaminose suggests that the isomerization step (**2** → **3**) proposed to be TylM3-catalyzed failed to occur. This is unlikely a gene expression problem since TylM2 was clearly expressed and capable of attaching quinovose to tylactone in the host. The recent in vitro study of the glycosyltransferase activity of DesVII revealed the dependence of DesVII on the "activator" protein, DesVIII, for activity.[8] Since TylM3 is a DesVIII homologue (33% identity), the above discovery suggests that TylM3 may not function as a 3,4-isomerase as proposed, but may be a partner protein in the TylM2-catalyzed glycosylation.[9] Thus, it is likely that not all the necessary genes to reconstitute the mycaminose pathway are present in pCM7b. In the absence of the 3,4-isomerase, mycaminose biosynthesis would not proceed beyond **2** in the KdesI/VII-pCM7b mutant, resulting in the incorporation of TDP-quinovose (**15**), the reduced form of the aborted intermediate **2**, in the final product **16** (Scheme 2, route A).

To locate the missing 3,4-isomerase gene, a detailed analysis of all open reading frames (ORFs) within the tylosin gene cluster was conducted. To our delight, a small previously unassigned ORF (0.44 kb), *tyl1a*, directly upstream of *tylB* (Figure 1), was found by a BLAST search to be homologous to an ORF in the *S*-layer polysaccharide biosynthetic gene cluster of *Aneurinibacillus thermoaerophilus*. The encoded protein of the latter gene had recently been shown to catalyze the conversion of **2** to TDP-3-keto-6-deoxy-D-galactose, the C-4 epimer of **3**.[10] To verify the function of *tyl1a*, a new construct, pCM21, containing *tylM3*, *tylM2*, *tylM1*, *tyl1a*, and *tylB* was prepared (Scheme 2, route B). When expressed in the KdesI/VII mutant, along with exogenously added **14**, two new compounds were found in the chloroform extracts of the culture broth. Their identities were determined by NMR and high-resolution MS analyses to be 5-*O*-mycaminosyl−tylactone (**6**) and 2′-glucosyl-5-*O*-mycaminosyl−tylactone (**17**).[7] Both are new macrolide derivatives. Compound **17** is likely derived from **6** by the action of a resistance glycosyltransferase present in *S. venezuelae*.[11] These data clearly demonstrate the success of in vivo reconstitution of the entire mycaminose pathway, providing compelling evidence for the assignment of Tyl1a as the TDP-4-keto-6-deoxy-D-glucose 3,4-isomerase that catalyzes the conversion of **2** to **3**.

Having established the biosynthetic pathway for mycaminose, it is now possible to explore the feasibility of converting the "glycosyl machinery" that produces desosamine to one that yields mycaminose. For this purpose, pCM30, carrying the *tyl1a* gene alone, was constructed. Upon expression in the KdesI strain of *S. venezuelae*, the desosamine pathway is expected to be transformed to a functional mycaminose pathway (Scheme 2, route C). The premise of this experiment is based on two earlier observations: *tylB* could complement a KdesV mutant,[12] and DesVI and TylM1 could accommodate each other's substrates.[4a] A small-scale culture of the KdesI-pCM30 mutant was grown from which six compounds were isolated and identified in the chloroform extracts of the culture broth. The two major products were determined by high-resolution MS and NMR analyses to be 3-*O*-mycaminosylated methynolide (**18**) and neomethynolide (**19**), and the third related metabolite was found by high-resolution MS analysis as 3-*O*-mycaminosyl-10-deoxymethynolide (**20**).[7] Three minor products were also identified by high-resolution MS as the mycaminosyl derivatives of novamethynolide (**21**), narbonolide (**22**), and pikronolide (**23**). These findings clearly demonstrated that a single gene substitution (*tyl1a* for *desI*) is sufficient to convert the desosamine pathway to a mycaminose pathway, and also showed that both DesV and DesVI exhibit substrate flexibility. Together, the two proteins were able to convert the Tyl1a product (**3**) to TDP-mycaminose (**5**).

In summary, the results reported herein firmly establish the catalytic role of Tyl1a as TDP-4-keto-6-deoxy-D-glucose 3,4-isomerase. This finding is significant because it assigns a function to an orphan ORF whose encoded protein catalyzes an important conversion in unusual sugar biosynthesis. Because its homologues likely catalyze similar isomerization reactions in sugar formation, their existence in a gene cluster will shed light on the encoded biosynthetic pathway. With this gene identified, the mycaminose pathway is now fully elucidated, adding a valuable addition to our collection of "glycosyl tools". The fact that nine new compounds (**6**, and **16**−**23**) were produced in this work clearly shows the applicability of these tools to the synthesis of new macrolide derivatives. Finally, we have demonstrated the facile conversion of one sugar pathway to another by exchanging selected "parts" (genes). The relative simplicity of this transformation is encouraging for efforts to construct diverse nucleotide sugars for glycodiversification of secondary metabolites.

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Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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