Mechanistic Studies of Desosamine Biosynthesis: C-4 Deoxygenation Precedes C-3 Transamination

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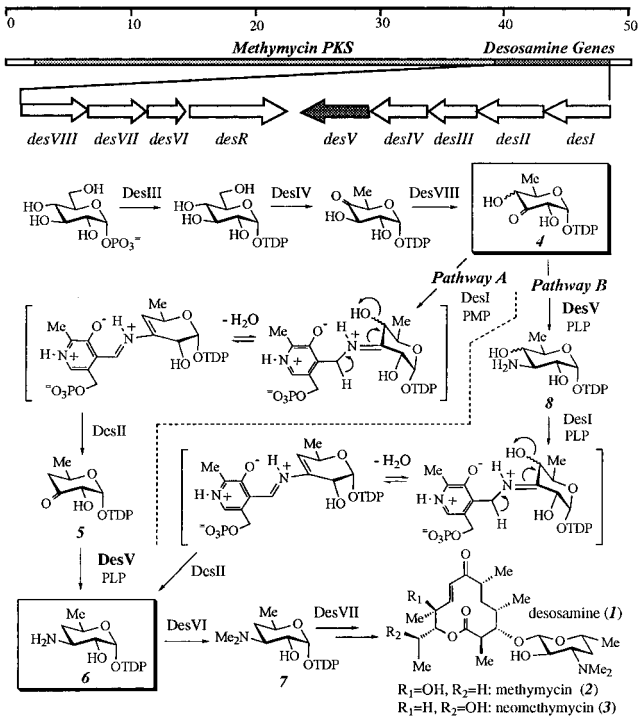
Desosamine (**1**) is a 3-amino-3,4,6-trideoxyhexose found in several macrolide antibiotics such as methymycin (**2**), neomethymycin (**3**), pikromycin, and erythromycin.[1] Since modifying the structure of the sugar component holds promise for varying and/or enhancing the biological activities of the parent antimicrobial agents,[2] a detailed understanding of the biosynthesis of desosamine is essential for developing suitable strategies to control, mimic, or alter its formation. Toward these goals, we have recently cloned and sequenced the entire desosamine biosynthetic gene cluster from the methymycin/neomethymycin producing strain, *Streptomyces venezuelae*.[3] As shown in Scheme 1, eight of the nine open reading frames (ORFs) in this region have been assigned to steps in the biosynthesis of TDP-D-desosamine (**7**), while the remaining ORF, *desR*, which encodes a macrolide β-glucosidase, appears to be involved in a glycosylation−deglycosylation self-resistance mechanism.[3a] These assignments are based on sequence similarities to other sugar biosynthetic genes, especially those derived from the erythromycin cluster that has been independently characterized by Gaisser et al.,[4a] Salah-Bey et al.,[4b] and Summers et al.[4c] Of particular interest in this cluster is the presence of two genes, *desI* and *desV*, both of which display sequence homology to coenzyme B_6-dependent catalysts.[5] The encoded proteins of these two genes have been assigned to catalyze two of the key reactions in this pathway—DesV may be an aminotransferase responsible for the C-3 transamination, whereas DesI is likely a dehydrase involved in the C-4 deoxygenation.[3,4]

Depending on the order of these two key steps, two possible pathways have been proposed for the biosynthesis of TDP-D-desosamine (**7**).[3,4] As shown in Scheme 1 pathway A, the C-4 deoxygenation, postulated to be mediated by the 4-dehydrase (DesI) and a putative reductase (DesII),[6] takes place on **4** to give **5**. This is followed by C-3 transamination catalyzed by DesV to afford **6**.[3,4c] Alternatively, the C-3 transamination catalyzed by DesV may occur first to generate 3-aminosugar intermediate **8**, which is then processed by the 4-dehydrase (DesI) and the reductase (DesII) to give **6** (Scheme 1, pathway B).[4a,b] The mechanisms proposed for C-4 deoxygenation in both pathways are essentially alike and are based on the well-established C-3 deoxygenation in the biosynthesis of 3,6-dideoxyhexoses.[7] However, the substrate specificity and the cofactor requirements for the 4-dehydrase are clearly different in each case.[8] Although isolation and characterization of these enzymes would provide direct evidence to distinguish these mechanistic possibilities, due to problems encountered in expressing the corresponding genes to give soluble proteins, an approach relying on gene deletion and phenotype correlation was adapted to determine the sequence of events in this pathway and to define the chemical nature of the substrate for the 4-dehydrase.

Scheme 1



First, a double crossover mutant of *S. venezuelae* (KdesV-41) in which the *desV* gene has been replaced by the thiostrepton resistance gene (*tsr*) was constructed.[9] This KdesV-41 mutant was first grown at 29 °C for 48 h in seed medium (100 mL) and

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(1) *Macrolide Antibiotics, Chemistry, Biology, and Practice*, Omura, S. Ed., Academic Press: New York; 1984.

(2) Weymouth-Wilson, A. C. *Nat. Prod. Rep.* **1997**, *14*, 99−110.

(3) (a) Zhao, L.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc*. **1998**, *120*, 9374−9375. (b) Xue, Y.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12111.

(4) (a) Gaisser, S.; Bohm, G. A.; Cortés, J.; Leadlay, P. F. *Mol. Gen. Genet.* **1997**, *256*, 239−251. (b) Salah-Bey, K.; Doumith, M.; Michel, J.-M.; Haydock, S.; Cortés, J.; Leadlay, P. F.; Raynal, M.-C. *Mol. Gen. Genet.* **1998**, *257*, 542−553. (c) Summers, R. G.; Donadio, S.; Staver, M. J.; Wendt-Pienkowski, E.; Hutchinson. C. R.; Katz, L. *Microbiology* **1997**, *143*, 3251−3262.

(5) The translated sequences of *desI* and *desV* exhibit modest similarity (24% and 23% identity, respectively) to that of the pyridoxamine 5′-phosphate (PMP)-dependent 3-dehydrase (E_1) in the 3,6-dideoxysugar pathway.[7] However, both *desI* and *desV* bear a higher translated sequence homology to that of *tylB* (36% and 59% identity, respectively), a putative pyridoxal 5′-phosphate (PLP)-dependent aminotransferase gene from the tylosin cluster.[14] Since DesV more closely resembles TylB, it has been proposed that DesV is an aminotransferase that effects C-3 transamination, whereas DesI acts like an E_1 mimic catalyzing C-4 deoxygenation in desosamine formation. However, it should be pointed out that not only is E_1 a PMP enzyme but it also contains a [2Fe−2S] cluster in the active site. The lack of a presumed iron−sulfur binding motif in DesI clearly reflects the distinction between DesI and E_1.

(6) While no significant homologues of DesII could be found in the database, its functional equivalent, EryCV, in the erythromycin cluster has been postulated to be involved in the C-4 deoxygenation.[4]

(7) (a) Liu, H.-w.; Thorson, J. S. *Annu. Rev. Microbiol.* **1994**, *48*, 223−256. (b) Kirschning, A.; Bechthold, A. F.-W.; Rohr, J. In *Bioorganic Chemistry Deoxysugars, Polyketides & Related Classes*: *Synthesis, Biosynthesis, Enzymes*; Rohr, J. Ed.; Springer: Berlin; 1997, 1−84.

(8) The C-3 dehydrase, E_1, in the 3,6-dideoxysugar pathway is a unique enzyme that uses PMP not PLP as cofactor.[5,7] Early studies have also shown that the highly conserved Lys residue of all coenzyme B_6-dependent catalysts is replaced by a His residue in E_1 (Pascarella, S.; Bossa, F. *Protein Sci*. **1994**, *3*, 701−705; Lei, Y.; Ploux, O.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4643−4654). Since the invariant lysine is conserved in the translated DesI sequence, DesI has been proposed to be a PLP enzyme.[4a,b] However, caution must be exercised in speculating the coenzyme form based solely on sequence information. If DesI is indeed a PLP enzyme, a prior transamination converting PLP to PMP may be necessary for pathway A to be operative.

(9) The *tsr* gene was introduced as a selection mark to facilitate the screening of the mutant strains with phenotype of thiostrepton resistance (Thio).

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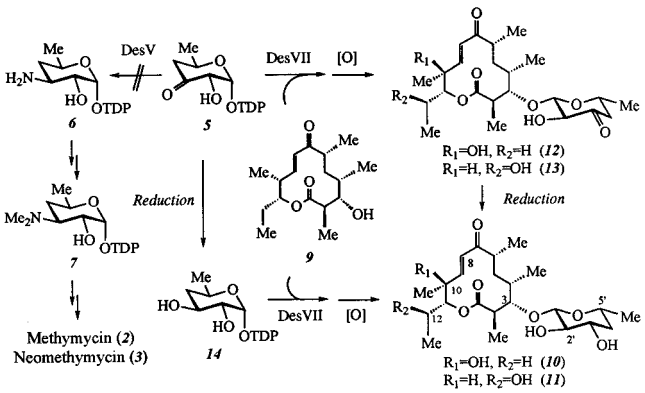
Scheme 2



then used to inoculate vegetative medium (3 L).[10] After incubation for another 48 h, the fermentation broth was centrifuged to remove the cellular debris and mycelia, and the supernatant was adjusted to pH 9.5 with concentrated KOH, followed by extraction with chloroform. As expected, no methymycin (**2**) or neomethymycin (**3**) was produced; instead, 10-deoxymethynolide (**9**) (360 mg)[11] and two new macrolides containing a 4,6-dideoxysugar, **10** (25 mg) and **11** (15 mg), were isolated.[12] The formation of **10** and **11** was somewhat surprising. As illustrated in Scheme 2, it is possible that **10** and **11** are derived from the predicted **12** and **13**, respectively, by a postsynthetic reduction of the attached ketosugar. It is also conceivable that a stereospecific reduction of **5** occurs first, followed by coupling of the resulting sugar **14** to the 10-deoxymethynolide **9**.[13] Although the exact order of this putative modification remains obscure, the absence of an amino functionality in the sugar component of these new products clearly corroborates the assignment of *desV* as an aminotransferase gene.[3,4] More significantly, these results are more consistent with pathway A. Since deletion of the *desV* gene leads to the incorporation of a 4-deoxysugar in the final products, one can conclude that deoxygenation at C-4 most likely occurs prior to the transamination at C-3.

In a separate experiment, a new mutant strain of *S. venezuelae* (KdesV-41X) was constructed from the KdesV-41 strain, in which the *tsr* gene was replaced by a *desV* equivalent, *tylB*, derived from the tylosin cluster.[14] The *tylB* gene is highly homologous to *desV*,[5,14] and its encoded protein is believed to be a PLP-dependent aminotransferase catalyzing the conversion of **4** to **8** in the mycaminose (a 3,6-dideoxy-3-*N*,*N*-(dimethylamino)-D-glucose) pathway. Since the natural substrate for TylB is **4**, which is also the predicted substrate for DesV in pathway B, such a complementation of *desV* by *tylB* in KdesV-41X is expected to restore most of the methymycin/neomethymycin production activity in this mutant strain if desosamine formation indeed follows pathway B. Interestingly, little **2** and **3** were found in the fermentation broth of KdesV-41X;[15] instead, the major products isolated were still **9**, **10**, and **11**. These findings suggest that TylB and DesV catalyze transamination of different substrates and thus lend further credence to pathway A in which C-4 deoxygenation proceeds prior to C-3 transamination.[16]

Although a full understanding of the actual mechanism of the C-4 deoxygenation must await further characterization of the DesI and DesII proteins, our current data have provided significant insights into the reaction sequence of the desosamine pathway. The fact that the *desV*-deletion mutant produces **10** and **11** carrying a modified deoxysugar also indicates that the glycosyltransferase (DesVII) of this pathway is capable of recognizing and processing sugar substrates other than TDP-desosamine (**7**).[4b,17] Thus, this work has also attested to the possibility for future production of hybrid glycosylated natural products by a combinatorial biosynthetic approach.

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Supporting Information Available: Characterization data of the new compounds **10** and **11**, including ^{1}H and ^{13}C NMR spectra, high-resolution FABMS results, and the complete spectral assignments (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(10) Cane, D. E.; Lambalot, R. H.; Prabhakaran, P. C.; Ott, W. R. *J. Am. Chem. Soc*. **1993**, *115*, 522−526.

(11) Lambalot, R. H.; Cane, D. E. *J. Antibiot*. **1992**, *45*, 1981−1982.

(12) Their structures were determined by spectral analyses and high-resolution MS. The data are summarized in the Supporting Information.

(13) Since both **10** and **11** are new compounds synthesized in vivo by the KdesV-41 mutant strain, the observed reduction might be a necessary step for self-protection (Cundliffe, E. C. *Annu. Rev. Microbiol*. **1989**, *43*, 207−233). Indeed, purified **10** and **11** are inactive against *Streptococcus pyogenes*.

(14) (a) Merson-Davies, L. A.; Cundliffe, E. *Mol. Microbiol*. **1994**, *13*, 349−355. (b) Gandecha, A. R.; Large, S. L.; Cundliffe, E. *Gene* **1997**, *184*, 197−203.

(15) Although studies of KdesV-41 mutant have provided compelling evidence supporting pathway A, we realize that the KdesV-41X mutant results are less conclusive since we cannot rule out that an inefficient heterologous expression of TylB may be the cause of the low production of **2** and **3** in the KdesV-41X mutant.

(16) In a recent report, the disruption of *eryCIV*, the *desI* equivalent in the erythromycin cluster, leading to the production of 4′-hydroxydesosaminyl erythromycin B as a minor product has been cited as evidence supporting pathway B.[4b] However, this result is not incompatible with pathway A if DesV can recognize **4** as an alternative substrate.

(17) A similar relaxed specificity toward sugar substrates has been reported for the glycosyltransferases in the biosynthesis of glycopeptide antibiotics (Solenberg, P. J.; Matsushima, P.; Stack, D. R.; Wilkie, S. C.; Thompson, R. C.; Baltz, R. H. *Chem. Biol*. **1997**, *4*, 195−202) and in daunorubicin (Madduri, K.; Kennedy, J.; Rivola, G.; Inventi-Solari, A.; Filippini, S.; Zanuso, G.; Colombo, A. L.; Gewain, K. M.; Occi., J. L.; MacNeil, D. J.; Hutchinson, C. R. *Nature Biotech*. **1998**, *16*, 69−74), erythromycin (Gaisser, S.; Bohm, G. A.; Doumith, M.; Raynal, M.-C.; Dhillon, N.; Cortés, J.; Leadlay, P. F. *Mol. Gen. Genet*. **1998**, *258*, 78−88), and methymycin/neomethymycin (Zhao, L.; Sherman, D. H.; Liu, H.-w. *J. Am. Chem. Soc*. **1998**, *120*, 10256−10257) pathways.