Study of C-4 Deoxygenation in the Biosynthesis of Desosamine: Evidence Implicating a Novel Mechanism

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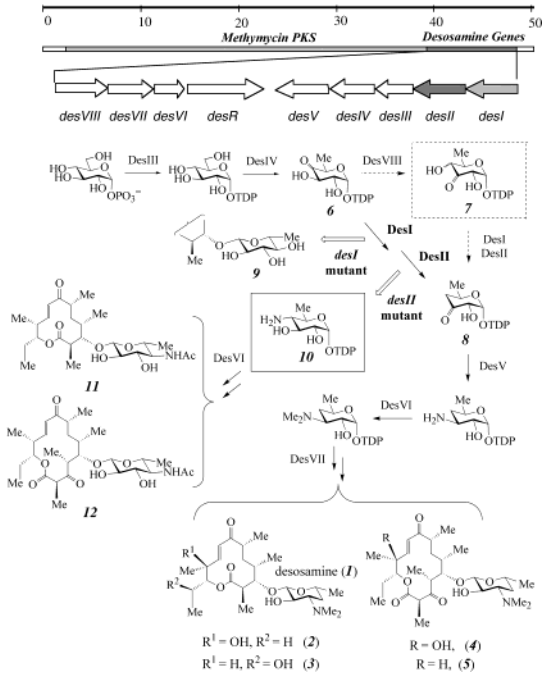
Carbohydrates are the focus of growing attention among biological molecules in recent years due to the increased appreciation of their vital roles in many physiological processes.[1] As components of many glycoconjugates, sugars, particularly the deoxysugars, contribute to a diverse repertoire of biological activities. Since modifying the structure of the appended sugars holds promise for varying or enhancing the biological activities of the parent glycoconjugates, there is considerable and continuing effort to explore how these unusual sugars are made in the producing organisms.[2] Such striving has led to the discovery of several elegant strategies evolved in nature for breaking the C−O bond of a hexose sugar. Thus, we can presently conclude that a sequence of α,β-dehydration followed by a hydride reduction is the mechanism for β-deoxygenation of a ketosugar precursor;[3,4] whereas a collaborative catalysis by a pyridoxamine 5′-phosphate (PMP)-dependent [2Fe−2S]-containing enzyme (E_1) and an NADH-dependent iron−sulfur flavoprotein reductase (E_3) is required for α-deoxygenation of a ketosugar substrate.[5]

While the mechanisms of C−O bond cleavage at C-2, C-3, and C-6 of a hexose have been fully established,[2] little is known about the mode of C−O bond scission at C-4 in making 4-deoxygenated sugars. Genetic studies on the biosynthesis of D-desosamine (**1**), a 3-(dimethylamino)-3,4,6-trideoxyhexose found in a number of antibiotics, resulted in the identification of the entire desosamine biosynthetic gene cluster from *Streptomyces venezuelae* (Scheme 1),[6] which produces methymycin (**2**), neomethymycin (**3**), pikromycin (**4**), and narbomycin (**5**). From this, eight open reading frames (*desI*−*desVIII*) within this cluster are suggested to be involved in desosamine biosynthesis including *desI* and *desII* that are assigned to be associated with the C-4 deoxygenation step.[6a,7] Since the translated sequence of *desI* shows high homology to B_6-dependent enzymes and is 24% identical to that of E_1, and the translated *desII* sequence contains a conserved motif of **C**XXX**C**XX**C** characteristic for a [4Fe−4S] center,[8] the C-4 deoxygenation has been postulated to follow a path similar to that catalyzed by E_1 and E_3.[6a,7] As illustrated in Scheme 1, the reaction may be initiated by a tautomerization step presumably catalyzed by DesVIII to convert **6**, a common precursor for 6-deoxyhexoses, to 3-keto-6-deoxyhexose **7**. DesI and DesII may then effect the removal of 4-OH from **7** to give the 3-keto-4,6-dideoxyhexose product (**8**) which has earlier been confirmed as the substrate of the next enzyme in the pathway, DesV.[9] This proposal is supported by the fact that 4-OH is retained in the appended sugar (D-quinovose, **9**) of the modified methymycin and pikromycin derivatives produced by the *desI* deleted mutant.[10] To learn more about this C−O bond cleavage event, we carried out targeted disruption of the *desII* gene and functional analyses of the DesI enzyme. Reported herein are the experimental results and the mechanistic implications on C-4 deoxygenation.

Scheme 1



To confirm whether DesII is a part of the C-4 deoxygenation machinery, a *S. venezuelae* mutant was generated in which the *desII* gene was replaced by the kanamycin resistance gene through homologous recombination of a plasmid containing the appropriate insert with the wild-type *S. venezuelae* chromosome.[11] This mutant strain was isolated and used for fermentation as previously described.[6a,12] It should be pointed out that E_1-catalyzed dehydration is a reversible reaction with equilibrium favoring the reverse direction,[13] and the reduction by E_3 is essential to drive the overall reaction to completion. Hence, if C-4 deoxygenation follows a

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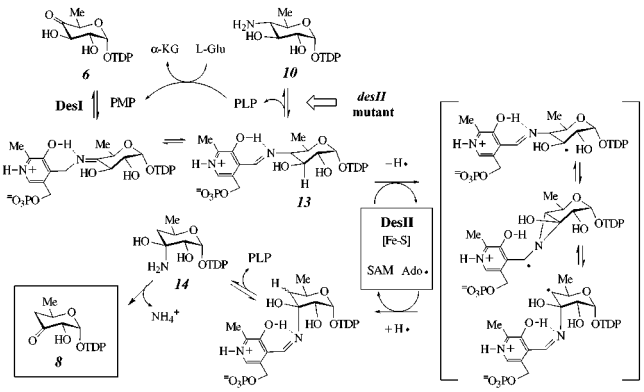
path similar to E_1/E_3 catalysis and DesII is an E_3-equivalent, disruption of the *desII* gene is expected to give a mutant with a phenotype that is identical to the *desI* mutant. Indeed, no wild-type antibiotics were found in the fermentation broth (6 L) of *desII* deleted mutant; instead, two macrolides containing an *N*-acetylated 4-aminosugar, **11** (2.4 mg) and **12** (1 mg), were obtained.[14] Compounds **11** and **12** are likely derived from the coupling of **10** and the respective aglycons, followed by *N*-acetylation (Scheme 1). However, it is also possible that *N*-acetylation of **10** occurs prior to its coupling to the aglycons. Regardless of the sequence of the events, the production of **11** and **12** clearly indicates that **10** must be accumulated in this *desII*-deleted mutant of *S. venezuelae*.

The above results provide a hint that DesI is a 4-aminotransferase, and 4-amination is the initial step of 4-deoxygenation. To verify the catalytic function of DesI, the *desI* gene was amplified by PCR and cloned into the pET-28b(+) expression vector (Novagen) with a His_6-tag at the *N*-terminus. The produced DesI protein was purified to near homogeneity by a Ni-NTA column (Qiagen) followed by FPLC on a MonoQ column. As judged by SDS-PAGE, the subunit M_r of DesI was estimated to be 45 kDa, which agrees well with the calculated molecular mass of 45 765 Da (plus the His_6 tag). Further analysis by size exclusion chromatography revealed a M_r of 95.6 kDa for DesI. Therefore, DesI exists as a homodimer in solution. The UV−vis spectrum of purified DesI is transparent above 300 nm; however, that of the more concentrated sample shows the presence of trace amount of PLP.

Interestingly, when the putative substrate, TDP-3-keto-6-deoxy-D-glucose (**7**)[15] was incubated with the purified DesI in the presence of L-glutamate, no consumption of **7** and no new product were discernible by HPLC analysis. On the contrary, when TDP-4-amino-4-keto-6-deoxy-D-glucose (**6**) was incubated with DesI under identical conditions, consumption of **6** (retention time = 4.52 min) and the formation of a new product (retention time = 3.87 min) were observed.[16] This new compound was purified by FPLC on a MonoQ column and characterized as the TDP-4-amino-4,6-dideoxy-D-glucose (**10**).[16] These results firmly establish that DesI only recognizes 4-hexulose **6** as the substrate and will not processes 3-hexulose **7** as previously surmised. These findings corroborate well with the *desII* gene disruption results. As a PLP-dependent 4-aminotransferase, a k_{cat} value of 56.2 ± 3.1 min^{-1} and a K_M value of 130 ± 4 μM for the sugar substrate **6** were also determined for DesI.

The fact that DesI, in the absence of DesII, catalyzes a transamination reaction on **6** to generate a 4-aminosugar product **10** calls for the modification of the previously proposed biosynthetic pathway for TDP-D-desosamine (Scheme 1). Clearly, the tautomerization of **6** to **7** is no longer a necessary step in the desosamine pathway. Furthermore, this implies that the mechanism of C-4 deoxygenation cannot be similar to that of the C-3 deoxygenation catalyzed by E_1/E_3.[2,5] Considering that DesI/DesII catalysis is initiated by the incorporation of a nitrogen functional group at C-4 (such as **13**), a 1,2-nitrogen shift from C-4 to C-3 to generate an aminal intermediate (such as **14**) may be the key step of C-4 deoxygenation. As illustrated in Scheme 2, elimination of either a water or an ammonium molecule from C-3 of **14** will generate the 3-keto-4,6-dideoxysugar product (**8**). There are enzymes capable of promoting 1,2-amino shift. The two best

Scheme 2



studied examples are ethanolamine ammonia lyase, an adenosylcobalamin (AdoCbl)-dependent enzyme that catalyzes the degradation of ethanolamine to ammonia and acetaldehyde,[17] and lysine 2,3-aminomutase which catalyzes the interconversion of L-lysine and L-β-lysine via 1,2-migration of the amino group.[17,18] The latter enzyme from *Clostridium subterminale* SB4 contains an iron−sulfur center and is PLP- as well as *S*-adenosylmethionine (SAM)-dependent. Both reactions are believed to involve a putative 5′-deoxyadenosyl radical which is generated by a reductive cleavage of SAM in lysine 2,3-aminomutase, or a homolytic cleavage of the Co−C bond of adenosylcob(III)alamin in ethanolamine ammonia lyase. This adenosyl radical then abstracts a hydrogen atom from the substrate to initiate the isomerization. Since DesI is a PLP enzyme and DesII has recently been identified as a member of radical SAM superfamily by sequence analyses,[19] the DesI and DesII enzymes may work together to catalyze a 1,2-amino migration analogous to that of lysine 2,3-aminomutase (see Scheme 2) to achieve C-4 deoxygenation.[20]

There is no doubt that this study has furnished compelling evidence indicating a new pathway for the biosynthesis of desosamine. Our results also allow the postulation of a new mechanism for C-4 deoxygenation, although the details of this process remain to be elucidated. A comparison of this new mechanism with that of C-3 deoxygenation clearly shows that nature has evolved diverse and elaborate strategies to pursue the removal of an α-OH from a ketohexose precursor in the biosynthesis unusual sugars. Taken together, studies conducted on the biosynthesis of deoxyhexoses have infused refreshing mechanistic insights into the general routes of biological deoxygenations. These findings are a good testament to the evolutionary diversity of biological C−O bond cleavage events.[21]

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Supporting Information Available: Conditions for DesI assay and spectral data of **10** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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