Biosynthesis

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Biosynthesis of TDP-D-Desosamine: Identification of a Strategy for C4 Deoxygenation**

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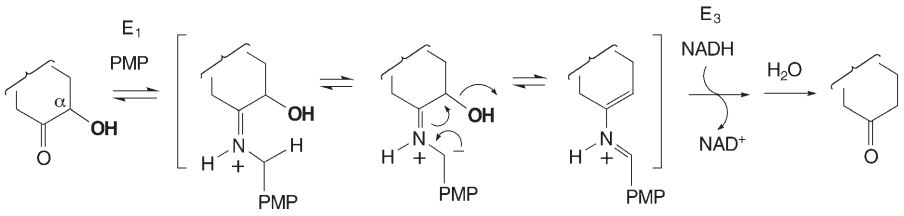
Dedicated to Professor Koji Nakanishi on the occasion of his 80th birthday

D-Desosamine (**1**), a 3-(dimethylamino)-3,4,6-trideoxyhexose found in a number of macrolide antibiotics including methymycin and pikromycin produced by *Streptomyces venezuelae*, plays an essential role in conferring biological activities to its parent aglycones.[1] Although numerous strategies for the biological C–O bond scission have been elucidated, little is known about the mechanism of deoxygenation at the C4 position in the formation of desosamine.[2] Insight into the mechanism of this key transformation is essential to fully establish the sequence of reactions in the desosamine pathway.

Past studies of the biosynthesis of various deoxyhexoses showed that the deoxygenation mechanism correlates with the position of the scissile C–O bond, either α or β to an activating group such as a keto group.[3] For example, a stepwise dehydration–reduction sequence catalyzed by a pyridoxamine 5′-phosphate (PMP)-dependent [2Fe-2S]-containing enzyme, E_1,[4] and an iron–sulfur flavoprotein reductase, E_3,[5] is the prototypical mechanism for α-deoxygenation of a ketosugar substrate (Scheme 1).[6] Hence, deoxygenation at C4 in the biosynthesis of D-desosamine could follow the α-deoxygenation path similar to that catalyzed by E_1 and E_3 starting from 3-keto-6-deoxyhexose (**3**) to give 3-keto-4,6-dideoxyhexose (**4**, Scheme 2, path A). This possibility is supported by the fact that the translated sequences of two genes, *desI* and *desII*, which are assigned to encode proteins involved in the C4 deoxygenation in the desosamine biosynthetic gene cluster,[7] are highly similar to B_6-dependent enzymes and those containing an iron–sulfur center, respectively.



Scheme 1. A prototypical mechanism for α-deoxygenation of a ketosugar substrate. E_1 and E_3 are iron- and sulfur-containing enzymes. PMP = pyridoxamine 5′-phosphate; NAD(H) = (reduced) nicotinamide adenine dinucleotide.

However, recent characterization of the purified DesI protein showed that it catalyzes the C4 transamination of **2** to generate TDP-4-amino-4,6-dideoxy-D-glucose (**5**; TDP = thymidine diphosphate) in the presence of pyridoxal 5′-phosphate (PLP) and L-glutamate.[8] Further sequence analysis revealed that DesII, which contains a [4Fe-4S] consensus motif (**C**XXX**C**XX**C**), belongs to the recently identified *S*-adenosylmethionine (SAM) superfamily of radical enzymes.[9] These two observations prompted a revision of the proposed biosynthetic pathway for TDP-D-desosamine. As depicted in Scheme 2 (path B), the DesI/DesII reaction may be initiated by the incorporation of a nitrogen-containing functional group from PMP at C4 (such as **6**), followed by a radical-induced 1,2-nitrogen shift (**6**→**7**→**8**→**9**) to yield an aminol intermediate (such as **10**). Subsequent elimination of an ammonium ion would afford the predicted product **4**. The regeneration of PMP may be facilitated by the transamination activity of DesI in the presence of L-glutamate.

The key mechanistic components of this reaction are reminiscent of those used in the reaction catalyzed by lysine 2,3-aminomutase (LAM), which is a [4Fe-4S]-containing enzyme that requires PLP and SAM for activity.[10] Catalysis by LAM is triggered by the abstraction of a hydrogen atom from the lysine–PLP adduct by the 5′-deoxyadenosyl radical (**11**; $AdoCH_2^{\bullet}$), which is formed by the reductive cleavage of SAM through involvement of the reduced [4Fe-4S] center.[11] A similar abstraction of a hydrogen atom, induced by the 5′-deoxyadenosyl radical, from **6** at C3 to give **7** may also be the key event of the C4 deoxygenation reaction. To test this hypothesis, we purified and studied the catalytic properties of DesII. Herein, we report the biochemical characterization of DesII and the mechanistic implications for the overall C4 deoxygenation reaction.

The *desII* gene was amplified from the genomic DNA of *S. venezuelae*[7b] by the polymerase chain reaction and cloned into the pET24b(+) vector at the *Nde*I and *Xho*I restriction sites.[12] The DesII protein with a His_6 tag at the C terminus was purified to near-homogeneity by using a Ni-NTA column followed by elution from a MonoQ column attached to an FPLC system.[13] A molecular mass of 48.4 kDa for DesII as estimated by gel filtration chromatography suggests that DesII, which has a calculated molecular mass of 54.265 kDa (including the His_6 tag), is a monomer in solution. Although purified DesII exhibits a broad absorption band between 400 and 500 nm ($\varepsilon_{420} = 4000\,M^{-1}cm^{-1}$), which is indicative of an iron–sulfur center,[14] titration showed the presence of only

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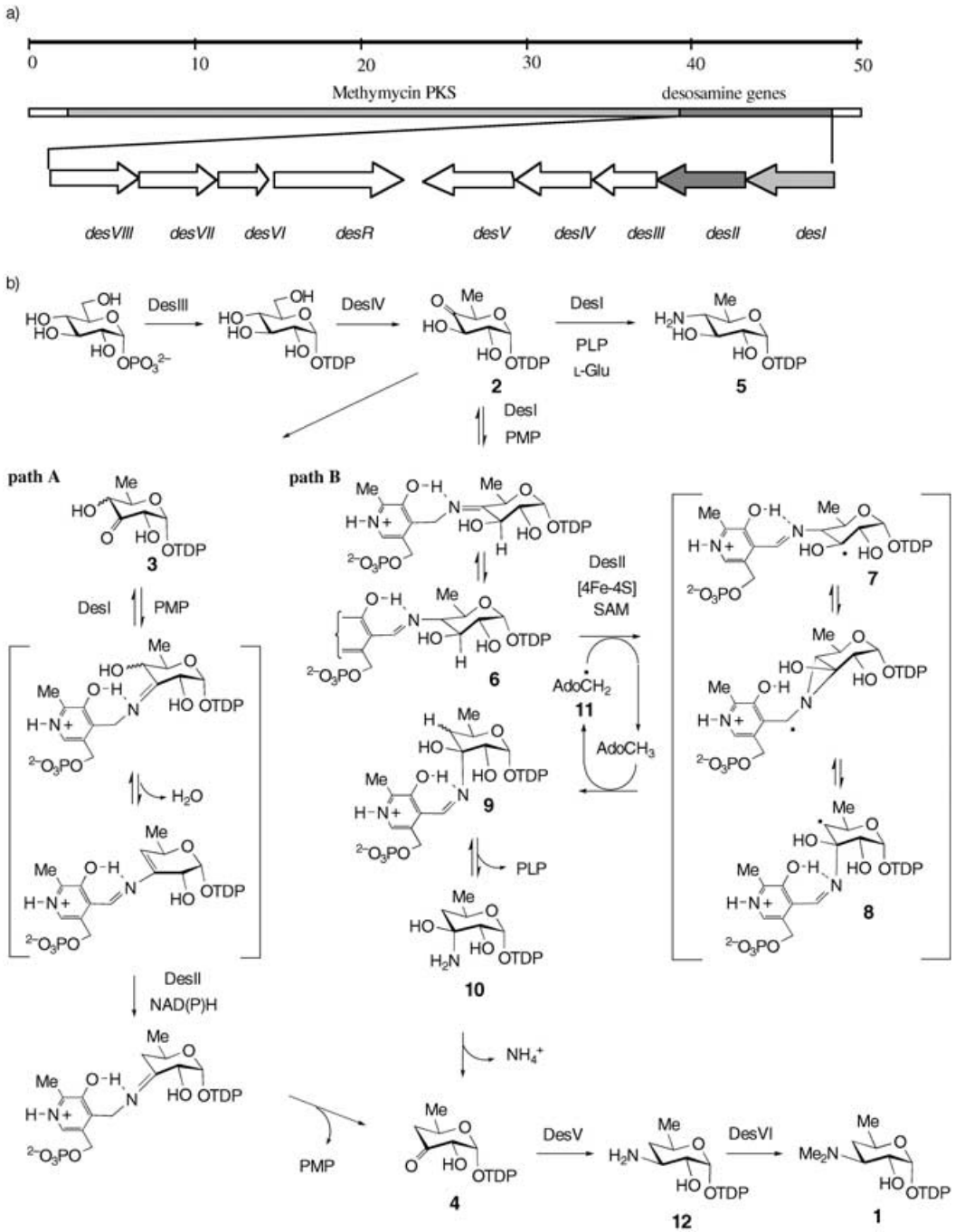


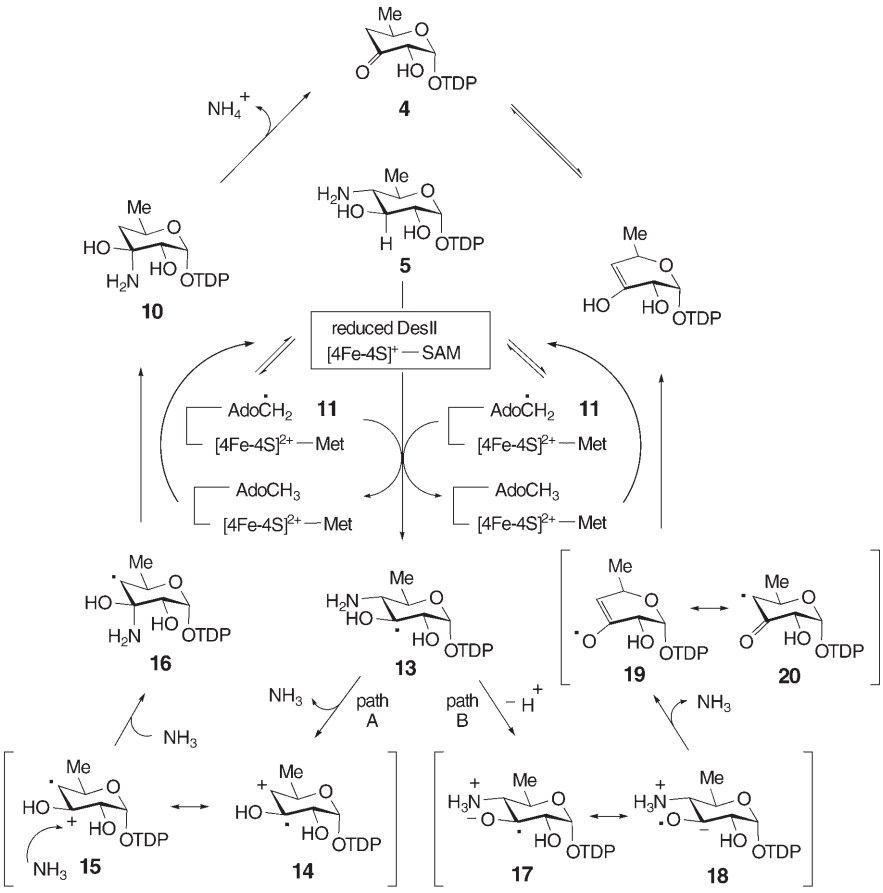
Scheme 2. a) Organization of the D-desosamine biosynthetic gene cluster. Scale indicates the length of DNA (kb). b) Possible mechanisms of deoxygenation at the C4 position in the biosynthesis of D-desosamine. TDP = thymidine diphosphate; L-Glu = L-glutamate; NAD(P)H = reduced nicotinamide adenine dinucleotide (phosphate).

0.6 equivalents of iron[15] and 0.5 equivalents of sulfur[16] per DesII monomer. Hence, most of the iron–sulfur cluster was depleted and/or decomposed during purification under aerobic conditions. Anaerobic reconstitution, carried out in the presence of a sixfold molar excess of Na_2S and $Fe(NH_4)_2(SO_4)_2$ in 100 mM Tris-HCl buffer, at pH 8 containing 5 mM dithiothreitol (DTT) at 18 °C,[17] led to holo-DesII, which contains nearly 4 equivalents of iron and 3.9 equivalents of sulfur atoms per subunit, with a broad absorption band at 420 nm ($\varepsilon_{420} = 9200\,M^{-1}cm^{-1}$). Both features are characteristic of a $[4Fe\text{-}4S]^{2+}$ cluster.[14]

To test the proposed mechanism of C4 deoxygenation, the purified and reconstituted DesII was treated with sodium dithionite anaerobically to reduce the $[4Fe\text{-}4S]^{2+}$ cluster to the $[4Fe\text{-}4S]^{+}$ state.[18] The reduced DesII was then incubated with DesI and **2**[8] in the presence of SAM, PMP (or PLP), and L-glutamate under anaerobic conditions.[19] HPLC analysis of the reaction mixture using a Dionex anion-exchange column

(4×250 mm)[20] revealed a new product with a retention time of 28.2 minutes which was not detected when dithionite or SAM was omitted from the incubation mixture. This new product was identified as **4** on the basis of its retention time, which was identical to that of a standard,[21] as well as high-resolution FAB-MS data (calcd for $[M-H]^-$: 529.0625, found: 529.0621). These results clearly demonstrate that SAM and the reduced $[4Fe\text{-}4S]^+$ cluster are required for DesII activity and they establish, for the first time, an essential role for DesII in the C4 deoxygenation reaction. The fact that the addition of DesV (in the presence of PLP and L-glutamate) to the above incubation mixture[22] led to the isolation of **12** as the final product[23] provided further evidence for the formation of **4** as a result of reaction with DesI/DesII.

To our surprise, when the reduced DesII was incubated by itself under anaerobic conditions with SAM and **5**, which was generated separately using DesI and **2** in the presence of PLP and L-glutamate,[8] compound **4** was again detected as the sole TDP–sugar product.[24] This finding, obtained in the absence of DesI, strongly suggests that the actual substrate of DesII is **5** and that the conversion of **2** into **4** proceeds in two steps, in which DesI catalyzes the C4 transamination of **2** to **5** and DesII carries out the C4 deamination of **5** to give **4**. The failure to establish the formation of a DesI–DesII complex in vivo by the yeast two-hybrid assay[25] is consistent with the observation that DesI and DesII function independently. Taken together, these results clearly demonstrate that DesII is a SAM-dependent deaminase, not a deoxygenase as previously surmised.



Scheme 3. Possible mechanisms of deoxygenation at C4 in the biosynthesis of D-desosamine. Generation of 5′-deoxyadenosyl radical (**11**) is expected to be the first part of the reaction. Met = methionine.

To account for these results, two possible mechanisms for the DesII-catalyzed reaction can be envisioned. As depicted in Scheme 3, generation of the 5′-deoxyadenosyl radical (**11**) is expected to be the first part of the reaction facilitated by the reduced $[4Fe\text{-}4S]^+$ center as found in other SAM–radical-dependent enzymes.[26] The actual chemical conversion may be triggered by abstraction of a hydrogen atom at C3 of **5** by **11** to give **13**. The mechanism for the subsequent transformation is less obvious, but may parallel the reaction catalyzed by the coenzyme B_{12} dependent ethanolamine ammonia lyase, which converts ethanolamine into ammonia and acetaldehyde.[27] As shown in Scheme 3 (path A), the key step may be a radical-induced deamination followed by the readdition of ammonia to the resulting cation radical intermediate (**14**/**15**), which is effectively a 1,2-amino shift (**13**→**14**/**15**→**16**→**10**), to form an aminol radical **16**.[28] Reclaiming a hydrogen atom from 5′-deoxyadenosine results in the formation of **10** and the regeneration of **11**, or more likely the reduced $[4Fe\text{-}4S]^+$–SAM complex.[29] Elimination of an ammonium ion from **10** would afford the desired product **4**. The reaction may also involve deprotonation of the 3-hydroxy group of **13** to yield a ketyl radical anion **17**,[30] whose resonance form **18** facilitates the β-elimination of the 4-amino group (Scheme 3, path B). The reaction catalyzed by (*R*)-2-hydroxyacyl-CoA dehydratase[31] in the fermentation of α-amino acids by anaerobic bacteria provides a precedent. The key step of the latter reaction involves ketyl radical anion induced C_β–O cleavage to expel a hydroxy group. Experiments to distinguish these mechanistic proposals are in progress.

In summary, the data reported herein show that DesII is a SAM-dependent [4Fe-4S]-containing enzyme and is directly responsible for the production of a key intermediate **4** in the biosynthesis of desosamine. In contrast to our previous proposal that DesI/DesII function as a pair to catalyze the α-deoxygenation of a 3-ketosugar substrate **3**, the C4 deoxygenation step is now established to proceed in two stages via an amino sugar intermediate **5**. These results are significant for two reasons: DesII has been identified as a unique deaminase, and the entire desosamine pathway has now been characterized (**2**→**5**→**4**→**12**→**1**). Although the mechanism of DesII catalysis remains elusive, utilization of 5′-

deoxyadenosyl radical (**11**) derived from SAM to abstract a hydrogen atom from **5** is believed to be an integral part of the reaction of DesII. Evidently, the mode of C4 deoxygenation by DesI/DesII is distinctly different from that of the E_1/E_3 paradigm. Currently, only a handful of SAM–radical enzymes have been characterized and all are involved in unusual biotransformations.[32] The fact that DesII is responsible for a radical-induced deamination further illustrates the catalytic versatility of this class of enzymes. Its participation with a transaminase (DesI) in an overall deoxygenation reaction underscores Nature's ingenuity in devising strategies for C–O bond scission.

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[12] Two primers were designed to amplify the *desII* gene by the polymerase chain reaction (PCR). The start primer, 5′-CGCG-**CATATG**ACCGCCCCCGCCCTTTCC-3′, contained an *Nde*I restriction site (in bold), and the stop primer, 5′-GCGC**CTCGAG**GCGCAGGAAGCC-3′, contained an *Xho*I restriction site (in bold). Amplification was carried out by using an initial incubation step at 95 °C for 3 min, followed by 30 cycles comprising incubation at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s. The PCR product was purified and ligated into the *Nde*I/*Xho*I sites of the vector pET24b(+). The resulting plasmid was used to transform *E. coli* BL21(DE3).

[13] The elution profile began with a linear gradient of 0 to 50% of buffer B (buffer A with 0.5 M NaCl) in buffer A (20 mM Tris-HCl, pH 7.5) for 25 min, followed by a gradient of 50–70% of buffer B in buffer A for 2 min. The flow rate was 3 mL min^{-1} and the detector was set at 280 nm. The DesII protein eluted at 55% buffer B.

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[17] Protein-bound iron was removed by incubating the as-isolated DesII in 100 mM Tris-HCl buffer (pH 8), containing 100 mM EDTA and 2 mM sodium dithionite, for 1 h at 25 °C. The apoprotein was loaded and eluted through a Sephadex G-25 column with the above Tris-HCl buffer and concentrated to 10 mg mL^{-1} by an Amicon concentrator with a YM10 membrane. This apo-DesII was incubated with a sixfold molar excess of Na_2S and $Fe(NH_4)_2(SO_4)_2$ in 1 mL of 100 mM Tris-HCl buffer (pH 8) containing 5 mM DTT for 3 h at 18 °C. A deaerated solution of EDTA (2 mM) was then added, and the incubation was continued for another 30 min. The reconstituted DesII was eluted through a Sephadex G-25 column as described above, and the green-gray protein fractions were pooled and concentrated to approximately 10 mg mL^{-1} by an Amicon concentrator with a YM10 membrane. All of the above operations were performed in an anaerobic chamber.

[18] Reduction of the [4Fe-4S] center of reconstituted DesII (0.19 mM) was achieved by using sodium dithionite (sixfold molar excess) prepared in 100 mM Tris-HCl buffer (pH 8) over 40 min. Reduction was conducted in an anaerobic chamber and monitored by the decrease of the absorbance at 420 nm.

[19] The activity assay was carried out anaerobically by mixing **2** (0.6 mM), L-glutamate (0.5 mM), pyridoxal 5′-phosphate (PLP; 0.14 mM), DesI (0.026 mM), reduced DesII (0.1 mM), and SAM (0.1 mM) in 100 mM Tris-HCl buffer (pH 8) in the presence of DTT (2 mM). The final volume was 1 mL. The reaction mixture was incubated at 25 °C for 3 h, and the reaction was quenched by filtering through an Amicon ultrafiltration cell equipped with a YM10 membrane to remove the enzymes. The reaction progress was monitored by HPLC.

[20] A linear gradient from 20 to 35% of eluent B (1M ammonium acetate, pH 7.0) in eluent A (H_2O) over 30 min gave a satisfactory separation. The flow rate was 0.6 mL min^{-1} and the detector was set at $\lambda = 267$ nm. The retention time for the substrate **2** is 13.8 min and that for DesII product **4** is 28.2 min, which is identical to that of the synthesized standard.[21]

[21] Compound **4** was prepared by incubating synthetic **12**[23] with purified DesV in the presence of PLP and α-ketoglutarate.

[22] To the reaction mixture prepared under conditions described in reference [19] (after 3 h incubation) were added L-glutamate (10 mM), PLP (0.8 mM), and DesV enzyme (0.1 mM). The resulting mixture was incubated at 25 °C for 30 min, and then passed through an Amicon ultrafiltration cell equipped with a YM10 membrane to remove the enzymes. The formation of the DesV product **12** was detected by HPLC;[20] the retention time of 3.7 min is identical to that of the synthesized standard.[23]

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[24] Typically, 1 mL of assay solution contained substrate **5** (0.5 mM), reduced DesII (0.1 mM), and SAM (0.1 mM) in 100 mM Tris-HCl buffer (pH 8) with 2 mM DTT. The assay mixture was incubated

anaerobically at 25 °C for 1 h, and then the reaction was quenched by removing the enzymes by ultrafiltration through a YM10 membrane (Amicon). The reaction progress was monitored by HPLC as described above.[20]
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[28] This 1,2-migration may be a stepwise transfer (**13**→**14**/**15**→**16**) or a direct transfer (**13**→**16**).
[29] As reclaiming a hydrogen atom from $AdoCH_3$ by **16** (or **19**/**20** in path B) is energetically unfavorable, stabilization of the resulting 5′-deoxyadenosyl radical (**11**) by reforming the $[4Fe\text{-}4S]^+$–SAM complex may provide the driving force to complete the catalytic cycle. A similar argument which invokes reversible cleavage of SAM by the [4Fe-4S] center has been proposed for the lysine 2,3-aminomutase reaction (D. Chen, C. Walsby, B. M. Hoffman, P. A. Frey, *J. Am. Chem. Soc.* **2003**, *125*, 11788 – 11789).
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