Biosynthesis

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Mechanistic Studies of the Radical *S*-Adenosylmethionine Enzyme DesII with TDP-D-Fucose**

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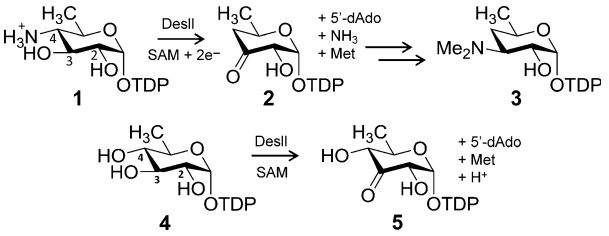
Abstract: *DesII is a radical S-adenosylmethionine (SAM) enzyme that catalyzes the C4-deamination of TDP-4-amino-4,6-dideoxyglucose through a C3 radical intermediate. However, if the C4 amino group is replaced with a hydroxy group (to give TDP-quinovose), the hydroxy group at C3 is oxidized to a ketone with no C4-dehydration. It is hypothesized that hyperconjugation between the C4 C–N/O bond and the partially filled p orbital at C3 of the radical intermediate modulates the degree to which elimination competes with dehydrogenation. To investigate this hypothesis, the reaction of DesII with the C4-epimer of TDP-quinovose (TDP-fucose) was examined. The reaction primarily results in the formation of TDP-6-deoxygulose and likely regeneration of TDP-fucose. The remainder of the substrate radical partitions roughly equally between C3-dehydrogenation and C4-dehydration. Thus, changing the stereochemistry at C4 permits a more balanced competition between elimination and dehydrogenation.*

The radical *S*-adenosylmethionine (SAM) enzyme DesII from *Streptomyces venezuelae* catalyzes the redox-neutral deamination of TDP-4-amino-4,6-dideoxy-D-glucose (**1**) to generate TDP-4,6-dideoxy-3-keto-D-glucose (**2**, TDP = thymidine diphosphate; Scheme 1).[1,2] In its biological context, the deamination of **1** is the key reaction in the biosynthesis of TDP-desosamine (**3**), which is an essential component of many macrolide antibiotics.[2–4] This deamination reaction is radical-mediated and is initiated by hydrogen atom abstraction from the substrate by a 5′-deoxyadenosyl radical. The latter is derived from the reductive homolysis of SAM by an active site $[4Fe\text{-}4S]^{1+}$ cluster and represents the hallmark of radical SAM enzymology.[5]



Scheme 1. Reactions catalyzed by DesII.

Two general mechanisms have been proposed for DesII-catalyzed deamination (Figure 1).[1,4] In both cases, the p orbital harboring the unpaired electron at C3 of the radical

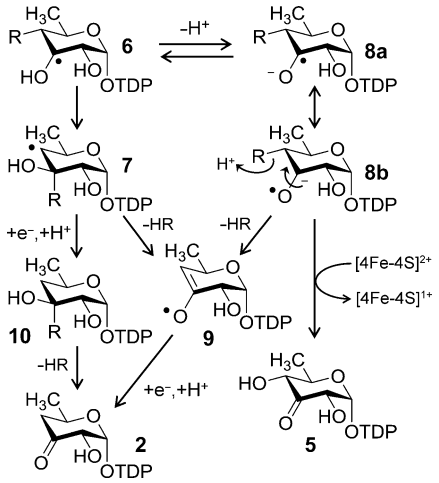


Figure 1. Possible reaction pathways for the substrate radical intermediate **6** during the DesII catalytic cycle. When R = NH_3^+, **6** may undergo either an elimination (e.g., **6**→**8**→**9**) or 1,2-migration (**6**→**7**→**9**/**10**) to produce **2**. When R = OH, **6** undergoes an oxidation, likely via the ketyl radical intermediate **8** to produce **5**.

intermediate **6** must overlap productively with the C–N σ-system at C4 in order to facilitate either 1,2-migration (**6**→**7**→**9**/**10**→**2**) or direct elimination (**6**→**8**→**9**→**2**) of the adjacent amino group. In this regard, DesII is highly reminiscent of ethanolamine ammonia lyase (EAL), which catalyzes the deamination of ethanolamine, albeit using a 5′-deoxyadenosyl radical produced from the homolysis of adenosylcobalamin rather than SAM.[6] Although the chemistry of DesII is also very similar to the dehydration of 1,2-diols by the B_{12}-dependent dioldehydratases,[7] no elimination of the C2 hydroxyl from **1** is observed during the catalytic cycle of DesII.

DesII can also accept TDP-D-quinovose (**4**), in which the C4 amino group of **1** is replaced with a hydroxy group, as a substrate. However, DesII does not catalyze elimination of the C4 hydroxy group from **4** to produce **2**, but rather catalyzes oxidation of the C3 hydroxy group to yield **5** (Scheme 1).[1] This second, dehydrogenase activity of DesII is

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analogous to the oxidation of 3-deoxy-*scyllo*-inosamine by the radical SAM enzyme BtrN from the butirosin biosynthetic pathway[8] and the dehydrogenation of a cysteine or serine residue to formylglycine catalyzed by anaerobic sulfatase maturating enzymes.[9] The dual capability of DesII to operate as a lyase in one instance and a dehydrogenase in another offers an ideal model system for investigating the subtleties of radical control within an enzyme active site.

Previous EPR investigation of the dehydrogenation reaction (**4**→**5**) catalyzed by DesII identified a C3 α-hydroxyalkyl radical intermediate in which the C–O bonds at both C2 and C4 are essentially orthogonal to the p orbital at C3.[10] While the structure of the radical intermediate during deamination has yet to be described, these observations led to the hypothesis that a difference in the binding configuration of the substrate radical is important for determining the partitioning of the C3-centered radical intermediate between the elimination and oxidation pathways (i.e., **6**→**2** versus **6**→**5**). It is proposed that whenever the configuration at C4 provides sufficient hyperconjugation between the C4 C–N/O σ system and the C3 p orbital, then elimination proceeds more rapidly than electron transfer to the $[4Fe\text{-}4S]^{2+}$ cluster, and lyase activity is observed. By contrast, if the configuration does not provide good overlap, then elimination is impeded. In this case, the more strongly reducing ketyl radical (**8b**) leads to faster reduction of the cluster compared to elimination of the C4 moiety, and dehydrogenase activity is observed.[2,11] Such a mechanism would help to explain why the C2 (in **1** and **4**) and C4 (in **4**) hydroxy groups are inert to dehydration during DesII catalysis, while α,β-dihydroxyalkyl radicals generated in solution through pulse radiolysis undergo rapid dehydration.[12] This working model implies that configurational inversion at C4 of the TDP-D-quinovose substrate radical (**6**, R = OH) could result in C4 dehydratase activity if such a stereochemical change permitted better hyperconjugation. This proposal can be tested by employing the C4 epimer of **4** (TDP-D-fucose (**12**); Scheme 3) as a potential substrate for DesII. Reported herein are the results and mechanistic implications of our studies of DesII using **12** as the substrate.

TDP-D-fucose (**12**) was prepared from TDP-D-glucose with 4,6-dehydratase RfbB followed by reduction with $NaBH_4$ as previously described (see the Supporting Information).[1,13] The consumption of both **12** and SAM was only observed upon prolonged incubation (hours) with a high concentration (10–20 μM) of DesII in the presence of $Na_2S_2O_4$. This corresponded to a specific activity of approximately 5×10^{-4} μmol min^{-1} mg^{-1} for the consumption of **12** versus 1 μmol min^{-1} mg^{-1} for the dehydrogenation of **4** at pH 8.0.[11] The much lower specific activity of DesII towards **12** helps to explain why it has not previously been recognized as a substrate.[1]

Three new products, in addition to 5′-deoxyadenosine, were observed by HPLC in the reaction of DesII with **12** (Figure 2). The product peak X with a retention time of 28.4 min was isolated and characterized by electrospray ionization mass spectrometry (ESI-MS). The detection of peaks at *m*/*z* 316.1 (positive ion X; Figure 3) and *m*/*z* 314.1 (negative ion) suggested that species X may be a sulfinate

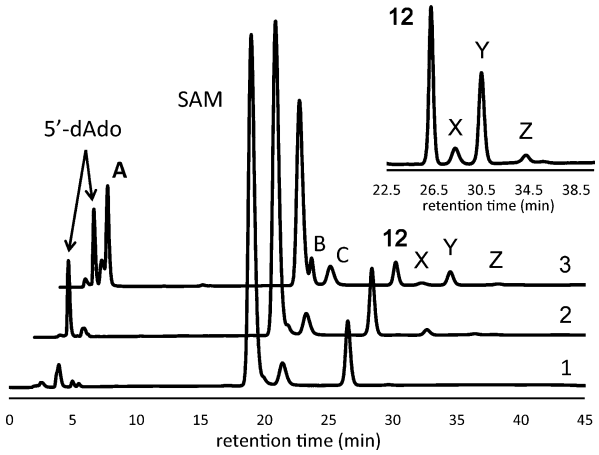


Figure 2. HPLC traces showing the consumption of TDP-D-fucose (**12**) and SAM in the presence of DesII and $Na_2S_2O_4$ at pH 8.0. Trace 1 was measured after 2 h without DesII. Traces 2 and 3 were measured after 30 min and 6 h in the presence of DesII, respectively. The retention times in traces 2 and 3 are shifted by 2 and 4 min compared to trace 1. Three new products (X, Y, and Z) are observed as shown in the inset (from Trace 3). The formation of 5′-deoxyadenosine (5′-dAdo) is also noted. Peak A corresponds to the methylthioadenosine decomposition product of SAM.[14] Peak B corresponds to thymidine monophosphate. Peak C indicates a contaminant in the SAM reagent.

adduct of deoxyadenosine (neutral mass: 315.1 Da). This hypothesis was supported by the observation of ESI-MS peaks at *m*/*z* 318.1 (positive ion X_d; Figure 3) and *m*/*z* 316.1 (negative ion) when [5′,5′-$^2H_2$]SAM was used instead of SAM. Further investigation showed that species X was also generated during prolonged co-incubation of DesII, SAM, and $Na_2S_2O_4$ in the absence of **12**; however, it was not formed if either SAM, DesII, or $Na_2S_2O_4$ was excluded from the

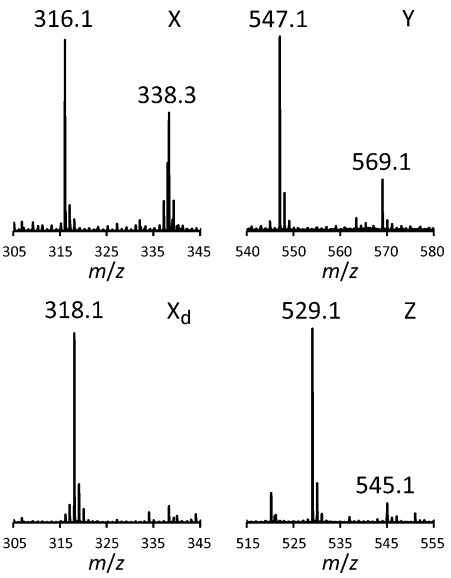
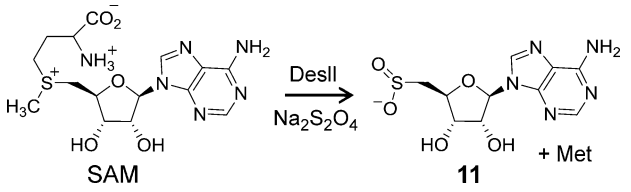


Figure 3. ESI-MS of isolated HPLC peaks X (positive ion), Y (negative ion), and Z (negative ion) from Figure 2. The spectrum in X_d (positive ion) corresponds to species X isolated from the reaction by using [5′,5′-$^2H_2$]SAM. The peak at *m*/*z* 338.3 likely corresponds to a known contaminant (i.e., erucamide) in the ESI-MS rather than the $[M+Na]^+$ peak of X.



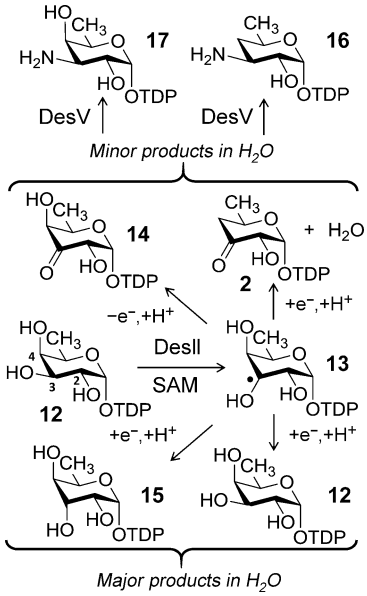
Scheme 2. Conversion of SAM to **11** in the presence of DesII and $Na_2S_2O_4$

reaction mixture. Based on these observations, species X was assigned as 5′-deoxyadenosyl-5′-sulfinic acid (**11**; Scheme 2).

The formation of sulfinate adducts has been reported in the reactions of two other radical SAM enzymes when $Na_2S_2O_4$ is used to maintain a reduced $[4Fe\text{-}4S]^{1+}$ cluster. For example, a mutant of spore photoproduct lyase has been shown to catalyze the production of a sulfinic acid adduct of dithymidine monophosphate.[15] The atypical radical SAM enzyme Dph2, which employs a SAM-derived 3-amino-3-carboxypropyl (ACP) radical rather than a 5′-deoxyadenosyl radical, has also been shown to produce a sulfinic acid derivative of ACP during turnover.[16] However, to our knowledge, the conversion of SAM into **11** in the presence of DesII and $Na_2S_2O_4$ is the first report of a sulfinate derivative of 5′-deoxyadenosine being generated by a radical SAM enzyme. The specific activity for the sulfination reaction is no greater than 2×10^{-3} μmol min^{-1} mg^{-1} in the absence of TDP-D-fucose and its rate of formation is reduced in the presence of the TDP-sugar. This result suggests that dithionite can access the DesII active site and intercept the 5′-deoxyadenosyl radical, especially in the absence of a sugar substrate.

Both of the remaining two product peaks in Figure 2 originated from TDP-D-fucose (**12**). ESI-MS analysis of the major product peak (Y, retention time 30.5 min) showed signals indicative of $[M-H]^-$ and $[M-2H+Na]^-$ ions at *m*/*z* 547.1 and 569.1, respectively (Figure 3). This result is consistent with an isomer of TDP-D-fucose; however, the HPLC retention time and relative inertness to reaction with DesII ruled out assignment as TDP-D-quinovose (**4**). Species Y was found to be sufficiently stable to permit collection for ^{1}H NMR analysis, and all coupling constants between protons on the hexose ring are relatively small (< 6 Hz), thus indicating an absence of *trans*-diaxial C–H bonds. This result implies a diaxial configuration of the vicinal hydroxyl groups at C3 and C4 and led to the assignment of species Y as TDP-6-deoxy-D-gulose (**15**), which is the C3-epimer of TDP-D-fucose. However, the ^{1}H NMR spectra of species Y exhibited significant contamination due to partial decomposition, and a standard of **15** was prepared in order to confirm the assignment by both HPLC coinjection and ^{1}H NMR spectroscopy (see the Supporting Information). The formation of TDP-6-deoxy-D-gulose from TDP-D-fucose implies that net H-atom return to the C3 radical intermediate (**13**; Scheme 3) of TDP-D-fucose is also possible in addition to dehydration and dehydrogenation (see below).

When the reaction was run in buffer containing at least 95% deuterium, ESI-MS analysis showed an approximately four-fold incorporation of deuterium versus protium into the



Scheme 3. Summary of reactions catalyzed by DesII when TDP-D-fucose (**12**) serves as the substrate.

product **15**. Furthermore, **15** went from being the major distinguishable product (ca. 80%) in H_2O to a minor product (< 30%) in D_2O, thus indicating a solvent deuterium kinetic isotope effect on the partitioning of **13** between the different routes of decomposition. The deuterium content of the residual TDP-D-fucose in the D_2O buffers was also investigated by mass spectrometry. Small but measurable increases in the deuteration of the residual substrate (**12**) were observed, thus suggesting that net H-atom transfer is possible to both faces of the C3 radical. These results indicate that **13** is solvent accessible, and the H atom transferred to C3 originates from a solvent-exchangeable source (see the Supporting Information).

The later eluting peak Z from the DesII reaction with TDP-D-fucose at 34.3 min co-eluted with the deamination product **2**. Furthermore, negative mode ESI-MS of the collected peak exhibited a signal at *m*/*z* 529.1 (Figure 3). This value is consistent with its assignment as the dehydration product **2**. To verify the identity of species Z as **2**, the DesII reaction with **12** was further treated with the transaminase DesV, which catalyzes the reductive amination of **2** in the presence of glutamate.[17,18] This resulted in the disappearance of peak Z and formation of a new HPLC peak that co-eluted with TDP-3-amino-3,4,6-trideoxy-D-glucose (**16**) as predicted (see the Supporting Information). These observations indicate that DesII is indeed capable of operating as a dehydratase.

The dehydratase activity of DesII was also investigated by using a DesII/DesV coupled reaction system. Under these conditions, however, TDP-3-amino-3,4,6-trideoxy-D-glucose (**16**) was formed in a roughly 1:1 ratio with an additional peak at a retention time also consistent with a TDP-aminosugar. Negative ion ESI-MS analysis of the new peak revealed a signal at *m*/*z* 546.1, thus suggesting the $[M-H]^-$ ion of TDP-3-amino-3-deoxy-D-fucose (**17**). To confirm this assignment, the 3,4-ketoisomerase FdtA from *Aneurinibacillus thermoaer-*

ophilus was used in combination with DesV and TDP-6-deoxy-4-keto-D-glucose to produce a standard of TDP-3-amino-3-deoxy-D-fucose (**17**).[19,20] This standard indeed co-eluted by HPLC with the second DesII/DesV product from the TDP-D-fucose reaction (see the Supporting Information). The fact that the dehydrogenation product TDP-3-keto-D-fucose (**14**) was not directly observed during the reaction of TDP-D-fucose with DesII alone may be attributed to the poor stability of 3-keto TDP-sugars, which tend to readily decompose to TDP and ketodihydropyrans.[21] The observation that both TDP-3-amino-3,4,6-trideoxy-D-glucose (**16**) and TDP-3-amino-3-deoxy-D-fucose (**17**) are generated in an approximately 1:1 ratio in the DesII/DesV reaction indicates that dehydration and dehydrogenation of TDP-D-fucose (**12**) compete with one another to an approximately equal extent and without racemization at C4.

In summary, despite being a poor substrate, TDP-D-fucose (**12**) can be recognized by DesII, whereupon it undergoes H-atom abstraction at C3 (**12**→**13**; Scheme 3). The fate of the resulting C3-centered radical intermediate **13** is of interest because of its inverted stereochemistry at C4 compared to TDP-D-quinovose (**4**). The majority of **13** is reduced to produce TDP-6-deoxy-D-gulose (**13**→**15**) through net H-atom transfer from a solvent-exchangeable source, and regeneration of TDP-D-fucose (**13**→**12**) likely also takes place through an analogous process. Therefore, the altered geometry at C4 of **12** compared to **1** and **4** appears to destabilize the enzyme–intermediate complex, thereby permitting access to the radical by the solvent or leading to the dissociation of the complex.

Of the fraction of the C3-radical intermediate **13** that does not undergo reduction to **15**/**12**, approximately 50% is oxidized to produce TDP-3-keto-D-fucose (**14**), a result consistent with the dehydrogenase activity of DesII towards **4**. The remaining 50%, however, undergoes elimination of the C4 hydroxy group in direct analogy to the DesII catalyzed deamination of **1**. This observation is mechanistically significant since the virtually exclusive partitioning of the C3 radical with substrates **1** and **4** towards either elimination or dehydrogenation, respectively, becomes roughly 1:1 upon changing the stereochemistry of the C4 center.

These results support a mechanistic hypothesis in which the stereochemical configuration at C4 is important for determining the fate of the substrate radical intermediate and whether DesII operates as a lyase or a dehydrogenase. This model implies that the substrate radical of TDP-4-amino-4,6-dideoxy-D-glucose (**6**, $R = NH_3^+$) assumes a different conformation in the DesII active site compared to the radical of TDP-D-quinovose (**6**, R = OH). This difference in conformation would reduce the dihedral angle between the C-NH_3^+ σ system and the p orbital at C3, thereby resulting in improved hyperconjugation and facilitating radical induced C–N bond cleavage during the deamination of **1**.

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