Biosynthesis

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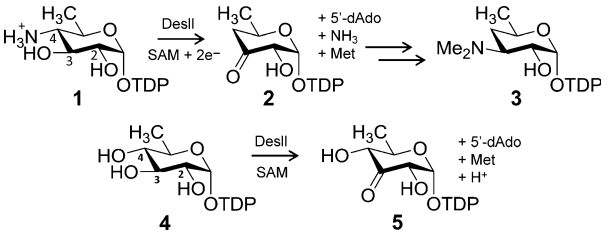
Mechanistic Studies of the Radical *S*-Adenosylmethionine Enzyme DesII with TDP-D-Fucose**

*Yeonjin Ko, Mark W. Ruszczycky, Sei-Hyun Choi, and Hung-wen Liu**

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

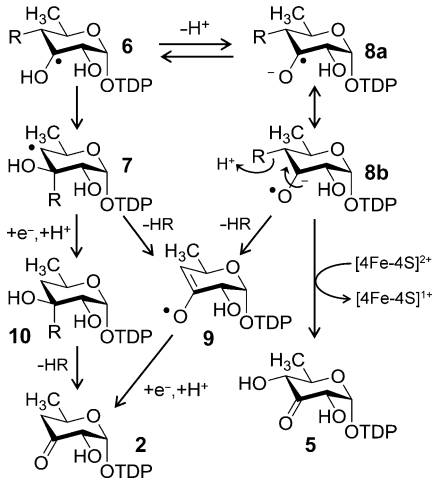


Figure 1. Possible reaction pathways for the substrate radical intermediate **6** during the DesII catalytic cycle. When R = NH_3^+, **6** may undergo either an elimination (e.g., **6**→**8**→**9**) or 1,2-migration (**6**→**7**→**9**/**10**) to produce **2**. When R = OH, **6** undergoes an oxidation,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

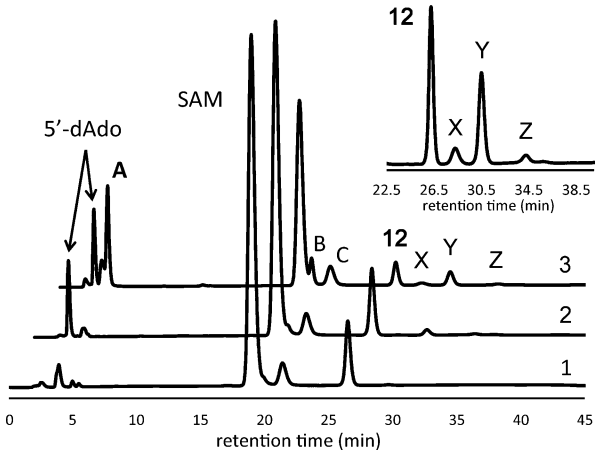


Figure 2. HPLC traces showing the consumption of TDP-D-fucose (**12**) and SAM in the presence of DesII and $Na_2S_2O_4$ at pH 8.0. Trace 1 was measured after 2 h without DesII. Traces 2 and 3 were measured after 30 min and 6 h in the presence of DesII, respectively. The retention times in traces 2 and 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

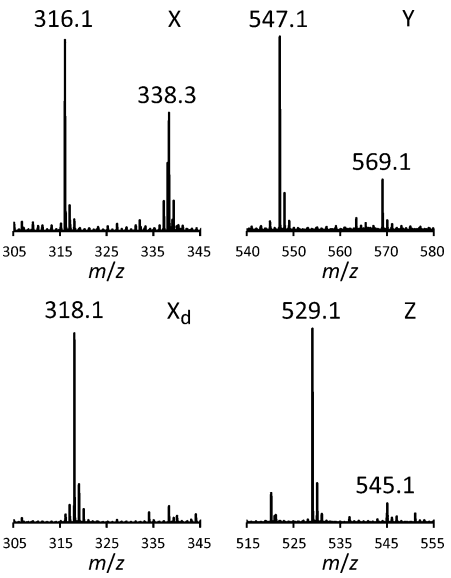
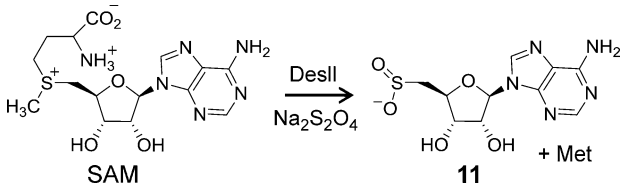


Figure 3. ESI-MS of isolated HPLC peaks X (positive ion), Y (negative ion), and Z (negative ion) from Figure 2. The spectrum in X_d (positive ion) corresponds to species X isolated from the reaction by using [5′,5′-$^2H_2$]SAM. The peak at *m*/*z* 338.3 likely corresponds to a known contaminant (i.e.,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



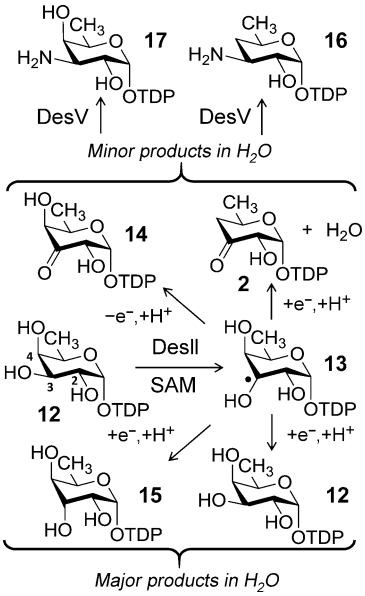
Scheme 2. Conversion of SAM to **11** in the presence of DesII and $Na_2S_2O_4$

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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Communications

Biosynthesis

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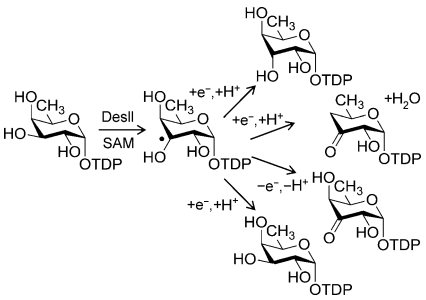
Mechanistic Studies of the Radical *S*-Adenosylmethionine Enzyme DesII with TDP-D-Fucose



What dictates the substrate's fate? DesII is a radical *S*-adenosylmethionine (SAM) enzyme that can catalyze either deamination or dehydrogenation depending on the substitution pattern of its substrate. By altering the stereochemistry of the dehydrogenation substrate, however, dehydration also becomes possible. This supports a model in which the fate of the substrate radical depends on its binding configuration in the enzyme active site.