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Mechanistic Investigation of the Radical *S*-Adenosyl-L-methionine Enzyme DesII Using Fluorinated Analogues

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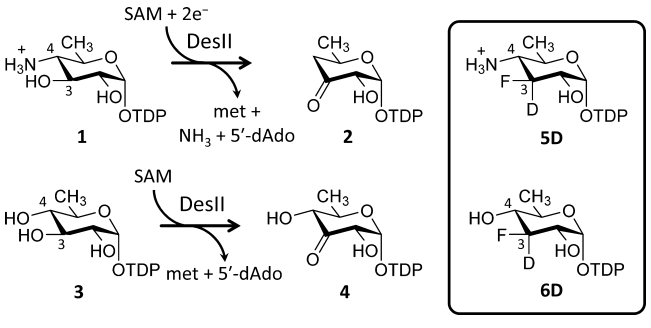
Supporting Information

ABSTRACT: DesII is a radical *S*-adenosyl-L-methionine (SAM) enzyme that can act as a deaminase or a dehydrogenase depending on the nature of its TDP-sugar substrate. Previous work has implicated a substrate-derived, C3-centered α-hydroxyalkyl radical as a key intermediate during catalysis. Although deprotonation of the α-hydroxyalkyl radical has been shown to be important for dehydrogenation, much less is known regarding the course of the deamination reaction. To investigate the role played by the C3 hydroxyl during deamination, 3-deutero-3-fluoro analogues of both substrates were prepared and characterized with DesII. In neither case was deamination or oxidation observed; however, in both cases deuterium was efficiently exchanged between the substrate analogues and SAM. These results imply that the C3 hydroxyl plays a key role in both reactions—thereby arguing against a 1,2-migration mechanism of deamination—and that homolysis of SAM concomitant with H atom abstraction from the substrate is readily reversible when forward partitioning is inhibited.

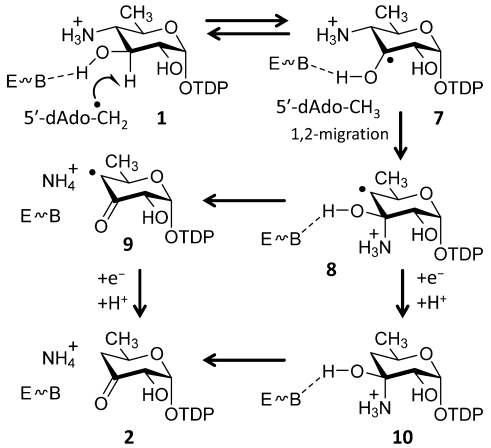
DesII is a radical *S*-adenosyl-L-methionine (SAM)[1] enzyme that is responsible for the key step in the biosynthesis of TDP-D-desosamine,[2,3] which is required for the glycosylation of various macrolide antibiotics such as erythromycin and pikromycin.[3−5] Specifically, DesII acts as a lyase to catalyze the radical-mediated deamination of TDP-4-amino-4,6-dideoxy-D-glucose (**1**) to yield TDP-4,6-dideoxy-3-keto-D-glucose (**2**) (see Scheme 1).[3,6] This chemistry is similar to the radical deamination reactions catalyzed by B_{12}-dependent ethanolamine ammonia lyase (EAL)[7] and the glycyl-radical enzyme CutC, which catalyzes the elimination of trimethylamine from choline.[8] In contrast, however, the DesII reaction is initiated by the reductive homolysis of SAM via electron transfer from an active-site $[4Fe-4S]^+$ cluster.[6] This produces a 5′-deoxyadenosyl radical (5′-dAdo-$CH_2^{\bullet}$) that subsequently abstracts the C3-hydrogen atom from **1** (see Schemes 2 and 3).[6] The resulting substrate radical **7** is thus activated for the elimination of ammonia; however, the mechanism by which this takes place is unclear.

Of particular interest is the role played by the C3-hydroxyl in the deamination of **1**. One hypothesis is that the C3-hydroxyl is not directly involved in the subsequent deamination. In this case, the key step is proposed to be a radical-induced 1,2-migration of the protonated C4-amino functionality to generate a C3-carbinolamine-C4-radical (**7** → **8**), as shown in Scheme 2.

Scheme 1. Reactions Catalyzed by DesII



Scheme 2. Deamination via 1,2-Migration



Intermediate **8** then either undergoes reduction to a carbinolamine followed by elimination of ammonia (**8** → **10** → **2**) or eliminates ammonia prior to the reduction (**8** → **9** → **2**). Such a 1,2-migration mechanism is analogous to the current models of B_{12}-dependent catalysis for EAL and diol dehydratase and is supported by electron paramagnetic resonance and isotope-labeling studies.[7,9,10] The 1,2-migration mechanism is also consistent with gas-phase computations on the EAL system, where the corresponding transition state was found to be energetically reasonable and, in most of the cases considered,

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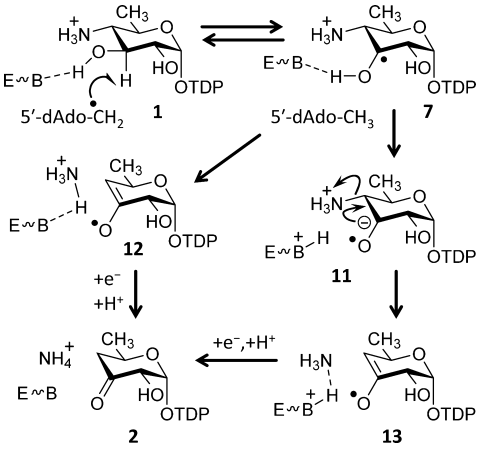
more favorable than mechanisms that do not involve direct migration.[11]

Despite evidence for the 1,2-migration mechanism, experiments involving the alternative DesII substrate TDP-D-quinovose (**3**) suggest that the C3-hydroxyl may actually play a more direct role in the deamination reaction. TDP-D-quinovose is the C4-hydroxy analogue of **1** and is readily accepted by DesII as a substrate.[12,13] As is the case with **1**, TDP-D-quinovose also undergoes C3-hydrogen atom abstraction within the DesII active site, which affords an α-hydroxyalkyl radical intermediate analogous to **7**;[14] however, the reaction does not result in the corresponding dehydration but rather C3-dehydrogenation.[6,12] This is in stark contrast to expectation because water is rapidly eliminated from 1,2-dihydroxyalkyl radicals generated via pulse radiolysis in bulk media.[15] Furthermore, the B_{12}-dependent diol dehydratases are well-known to catalyze analogous radical-mediated dehydration reactions rather than dehydrogenations.[9,10] Interestingly, kinetic isotope effect measurements indicated that deprotonation of the C3 α-hydroxyalkyl radical of **3** to generate a ketyl radical intermediate is necessary to facilitate electron transfer to the $[4Fe{-}4S]^{2+}$ cluster and thus complete the oxidation of **3** to **4**.[13] In light of these results, there is good reason to believe that the DesII active site contains a residue that can deprotonate the C3 α-hydroxyalkyl radical of whichever substrate is bound.

These observations suggest the alternative deamination mechanism shown in Scheme 3, in which the amino group is

Scheme 3. Deamination via Direct Elimination

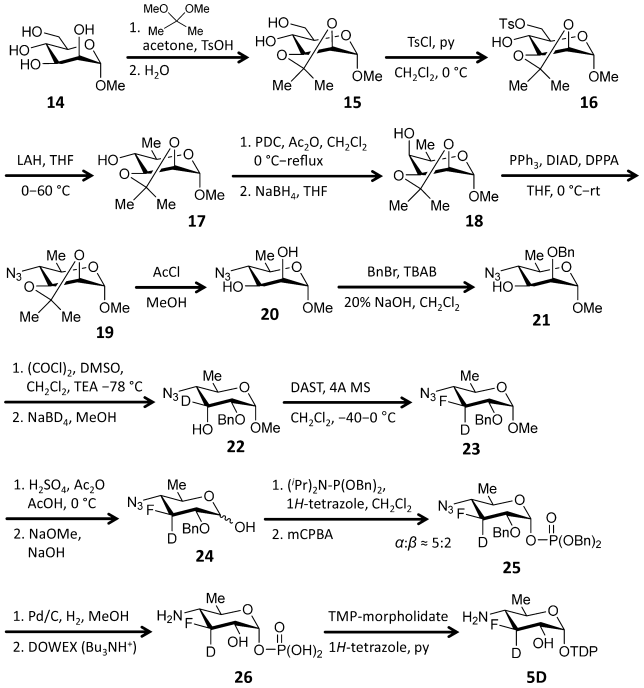


eliminated from C4 directly following the deprotonation of **7** to form a ketyl radical intermediate (i.e., **11**). Despite parallels with the dehydrogenation reaction (i.e., **3** → **4**), such an E1cB-type elimination of NH_3 (**11** → **13**) conflicts with the hypothesis that the formation of a strongly reducing ketyl radical promotes electron transfer back to the $[4Fe{-}4S]^{2+}$ cluster.[16] However, a process wherein the NH_3 leaving group accepts a proton from the C3 α-hydroxyalkyl radical in concert with its elimination (i.e., **7** → **12**) not only would resolve this conflict but also is consistent with computations that imply a similar activation energy compared with migration.[11] In this case, the putative active-site base may operate to facilitate the proton transfer as shown in Scheme 3, though a general acid may alternatively serve as a proton donor counterpart to the base.

One way to evaluate these mechanistic alternatives (i.e., 1,2-migration vs direct elimination) is to consider the effect of replacing the C3 hydroxyl with a less nucleophilic moiety that cannot be easily oxidized. To this end, we designed and synthesized C3-fluorinated **5** as a probe to test the importance of the C3-hydroxyl during DesII catalysis. Because of the increased electronegativity of a fluoro substituent,[17] its presence should impede the redistribution of electron density required for direct elimination (Scheme 3). In this case, deamination of **5** would not be observed. In contrast, the C3-fluoro group should have less impact on a radical-induced 1,2-migration of the amino group (Scheme 2). The result would be a 3-amino-3-fluoro species that subsequently decomposes to give **2**. We also prepared the analogue **6** to determine the effect of C3-fluoro substitution on the DesII-catalyzed oxidation reaction. Deuterium was introduced at C3 of both analogues in order to monitor C3-radical generation even in the absence of net turnover. Our data indicate that the C3-hydroxyl is indeed required for both the deamination and oxidation reactions. Reported herein are our observations with these compounds in the DesII system along with a discussion of the mechanistic implications.

The deuterated 4-amino-3-fluoro substrate analogue **5D** was prepared according to Scheme 4. The protected azide **19** was

Scheme 4. Synthesis of 4-Amino-3-fluoro Analogue 5D



obtained from methyl α-D-mannopyranoside (**14**) in a manner similar to a previously reported synthesis.[18] Intermediate **17** was sequentially oxidized and reduced to invert the stereochemistry at C4 (**17** → **18**) prior to installation of an azide moiety using a Mitsunobu-type azidation reaction (**18** → **19**). Acidic hydrolysis of isopropylidene **19** and selective benzyl protection of the C2-hydroxyl (**20** → **21**)[19] permitted the equatorial introduction of deuterium at C3 via Swern oxidation followed by $NaBD_4$ reduction (**21** → **22**).[20] Epimerization at C2 occurred during this reaction. Fluorination of **22** using diethylaminosulfur trifluoride (DAST) gave **23** with inversion of stereochemistry at C3.[20] This was followed by conversion to dibenzyl phosphate **25** via a glycosyl phosphite intermediate with the α-anomer as the major product. Hydrogenolysis of **25** led to benzyl deprotection and reduction of the C4-azido moiety to give **26**. Coupling of **26** with TMP-morpholidate provided **5D**.

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Compound **6D** was prepared in a similar manner (see the Supporting Information (SI)).

When **5D** or **6D** (250 μM) was incubated at room temperature for up to 8 h in the presence of 300 μM SAM, 1 mM $Na_2S_2O_4$, 25 mM 3-[4-(2-hydroxyethyl)piperazin-1-yl]-propane-1-sulfonate (EPPS) buffer (50 μL, pH 8.0) and DesII (3.7 to 30 μM), no consumption of the corresponding fluoro analogue was observed by HPLC (see Figure 1 and Figure 1S in the SI). Changes in the analogue concentration (0.25 to 2 mM) had no significant impact on the appearance of the HPLC traces (data not shown). Furthermore, there was no visible development of new HPLC peaks in the 30−40 min region, where the deamination and dehydration products (i.e., **2** and **4**) are expected to elute.[6,12] However, slow consumption of SAM was noted in both cases. The disappearance of SAM was dependent on both the enzyme concentration (Figure 1A) and the incubation time (Figure 1B) and was accompanied by the appearance of a new HPLC peak with a retention time of 27.3 min. Collection and electrospray ionization mass spectrometry (ESI-MS) analysis of this new peak demonstrated a signal at m/z 316.1 (positive ion), which is consistent with formation of 5′-deoxyadenosylsulfinate (5′-dAdo-SO_2H, predicted $[M + H]^+$ m/z 316.07). The generation of 5′-dAdo-SO_2^- during prolonged incubations of SAM and $Na_2S_2O_4$ with elevated concentrations of DesII has been reported previously[21] and implies that formation of the 5′-deoxyadenosyl radical indeed occurs in the reaction. This was further substantiated by the observation of low levels of 5′-deoxyadenosine at retention times earlier than 5 min.

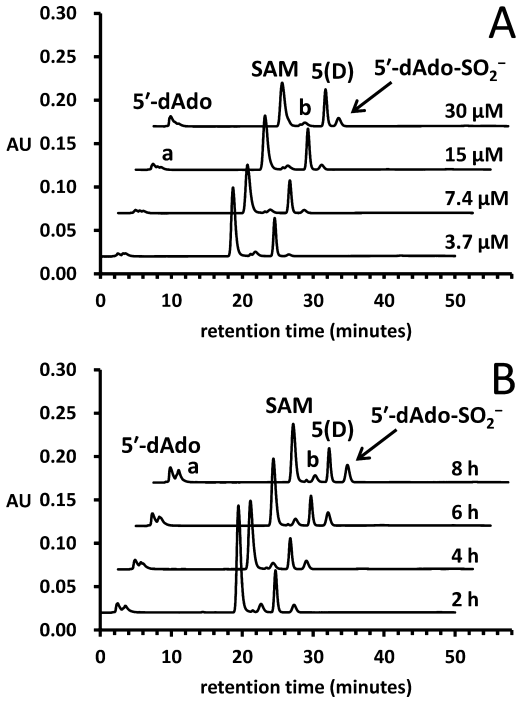

Figure 1. HPLC traces for incubations involving **5D**, SAM, and DesII in the presence of $Na_2S_2O_4$. Reactions were run anaerobically at room temperature in 25 mM EPPS buffer (pH 8.0). (A) Constant-time incubations of 2 h with variable DesII concentration (3.7−30 μM, indicated on each trace) and 250 μM **5D**, 300 μM SAM, and 1 mM $Na_2S_2O_4$. (B) Variable-time incubations of 2−8 h (indicated on each trace) with 3.7 μM DesII, 250 μM **5D**, 300 μM SAM, and 1 mM $Na_2S_2O_4$. Methylthioadenosine also contributes to the peaks at early retention times (peak a, <5 min),[23] and the minor peaks b correspond to thymidine monophosphate (TMP), impurities in the SAM preparation, and 3-*epi*-**5**.

To investigate this possibility, the DesII reactions with **5D** and **6D** were monitored for deuterium exchange between the fluoro analogues and SAM. Reaction mixtures containing 600 μM **5D** or **6D**, 300 μM SAM, 500 μM $Na_2S_2O_4$, and 20 μM DesII in 400 μL of 25 mM EPPS buffer (pH 8.0) were prepared. The reactions were carried out under anaerobic conditions at 30 °C. At 2 h intervals, additional fresh $Na_2S_2O_4$ was added and a 100 μL aliquot was removed. HPLC was then used to isolate the residual TDP-sugar substrates and SAM, which were subsequently analyzed by ESI-MS. Deuterium enrichment of each species was determined from the relative MS peak intensities after correction for natural-abundance ^{13}C.

Analysis of the changes in isotopic enrichment as a function of reaction time demonstrated that both **5D** and **6D** underwent significant depletion in deuterium content while at the same time SAM was enriched in both the mono- and dideuterated isotopologues (see Figure 3S). The exchange reactions with 20 μM DesII began to plateau within the first 2 h of incubation. Furthermore, at the 8 h time point, the fractional concentrations of monodeuterated **5** and **6** were 50% and 60%, respectively. Given the initial 2:1 ratio of the deuterated substrate isotopologue (one exchangeable hydrogen) and SAM (two exchangeable hydrogens), these values approach the expected equilibrium value of 50%, assuming that there is little difference in the isotopic fractionation factor[24] associated with the C3 position of the substrate and the 5′ position of SAM. Likewise, the expected enrichments of mono- and dideuterated SAM are 50% and 25%, respectively, which are similar to the values of ca. 50% and 20% observed at 8 h in the reactions with both C3-fluoro analogues. Finally, analysis of the 5′-deoxyadenosine produced demonstrated greater than 95% monodeuteration at early time points in the reaction.

The observation of efficient hydrogen exchange between the C3-fluoro analogues and SAM in the presence of DesII indicates that both substrate analogues undergo C3-hydrogen atom abstraction by the 5′-deoxyadenosyl radical generated in the active site. Careful inspection of HPLC traces from the reaction with **5D** also revealed a minor peak (<5%) consistent with 3-*epi*-**5** by ESI-MS (see Figure 2S). This minor product was not isotopically enriched beyond natural abundance and can account for the observed low levels of monodeuterated 5′-deoxyadenosine in these experiments. The epimerization likely results from reduction of the C3-radical intermediate **7** by a solvent-derived H atom equivalent, as has been previously reported in the case of TDP-D-fucose.[21]

The inability of **5D** and **6D** to undergo elimination or oxidation is thus not due to a failure to generate the substrate radical but is a consequence of the nonreactivity of the C3-fluoroalkyl radicals within the DesII active site. These results are mechanistically significant for three reasons. First, the fact that formation of a C3-radical adduct of **5** does not prompt deamination disfavors a mechanism involving a radical-induced

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1,2-migration of the amino group. Second, the results further substantiate the hypothesis that an increase in oxidation potential of the substrate radical is required for oxidation, since the ionization energy of a fluoromethyl radical is approximately 1 eV greater than that of a hydroxymethyl radical.[17,25] Third, the apparent equilibration of deuterium between the 3-fluoro analogues and SAM indicates that reductive homolysis of SAM and H atom abstraction from the substrate are readily reversible. Previous reports have provided evidence for this possibility under turnover conditions,[6,26,27] and enzymes such as lysine amino mutase,[28] spore photoproduct lyase,[29] and QueE[30] are well-known to regenerate SAM as part of their catalytic cycles. However, the present results suggest that the reverse partitioning of the substrate radical, methionine, and 5′-deoxyadenosine to regenerate SAM and the substrate (i.e., **7** → **1**) can become the dominant course of the reaction when forward partitioning (i.e., **7** → **8**, **11**, or **12**) is impeded.

Taken together, these results indicate that the mechanism of the DesII-catalyzed deamination reaction appears to be most consistent with that shown in Scheme 3. Upon formation of the ternary Michaelis complex between DesII, SAM, and **1**, SAM is reduced concomitant with C3-hydrogen atom abstraction from the substrate to produce the initial substrate radical **7**. This process is reversible, and the 5′-deoxyadenosyl or substrate radical may also be quenched unproductively to result in uncoupling, sulfinate formation, or C3-epimerization. However, these latter pathways are relatively minor in the case of DesII unless the substrate exhibits significant structural perturbations.[21]

The subsequent reaction of **7** depends on the presence of an ionizable hydroxyl group at C3, since radical-induced deamination of **5** does not take place. Instead, deamination likely proceeds via a mechanism of direct elimination wherein the proton from the C3 α-hydroxyalkyl radical is transferred to the amine leaving group at C4 (i.e., **7** → **12**). This may be mediated by the putative active-site base and likely proceeds in a concerted manner to avoid possible dehydrogenation due to the formation of a ketyl radical (i.e., **11**) as a discrete intermediate.[13] The catalytic cycle is then completed upon reduction of enol radical **12** by an external electron donor in a process that has yet to be clarified. Overall, the use of C3-fluoro analogues to investigate the DesII reaction has shed significant light on its mechanism of catalysis and radical SAM enzymes in general.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, enzyme assays, and spectroscopic characterization of all compounds discussed. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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