Biosynthesis

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Mechanistic Insights into the Radical *S*-adenosyl-L-methionine Enzyme NosL From a Substrate Analogue and the Shunt Products

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Abstract: *The radical S-adenosyl-L-methionine (SAM) enzyme NosL catalyzes the transformation of L-tryptophan into 3-methyl-2-indolic acid (MIA), which is a key intermediate in the biosynthesis of a clinically interesting antibiotic nosiheptide. NosL catalysis was investigated by using the substrate analogue 2-methyl-3-(indol-3-yl)propanoic acid (MIPA), which can be converted into MIA by NosL. Biochemical assays with different MIPA isotopomers in D_2O and H_2O unambiguously indicated that the 5′-deoxyadenosyl (dAdo)-radical-mediated hydrogen abstraction is from the amino group of L-tryptophan and not a protein residue. Surprisingly, the dAdo-radical-mediated hydrogen abstraction occurs at two different sites of MIPA, thereby partitioning the substrate into different reaction pathways. Together with identification of an α,β-unsaturated ketone shunt product, our study provides valuable mechanistic insight into NosL catalysis and highlights the remarkable catalytic flexibility of radical SAM enzymes.*

NosL is a radical *S*-adenosyl-L-methionine (SAM) enzyme that catalyzes a remarkable carbon-chain rearrangement of L-tryptophan (**1**) to produce 3-methyl-2-indolic acid (MIA, **2**; Figure 1),[1] a key intermediate in the biosynthesis of a clinically interesting thiopeptide antibiotic nosiheptide.[2] NosL-catalyzed MIA synthesis is initiated by hydrogen abstraction by a 5′-deoxyadenosyl (dAdo) radical, which results in fragmentation of the Cα–Cβ bond of L-tryptophan (**1**) to generate dehydroglycine (**3**) and an indolyl radical **4**, and this mechanism has been supported by the identification of the shunt product 3-methylindole (**5**) in an in vitro assay (Figure 1A).[1b,3] Similar Cα–Cβ bond lyase activity has also been observed in the catalysis of the hydrogenase-maturating enzyme HydG,[4] thiamine biosynthesis protein ThiH,[5] and the F_{420} biosynthesis protein CofG;[6] all of these enzymes cleave the Cα–Cβ bond of L-tyrosine.

However, unlike the tyrosine lyases, NosL catalysis involves a unique carbon-chain recombination process after Cα–Cβ bond cleavage, with the final outcome being release of the nitrogen atom and α-carbon of L-tryptophan as ammonia and formaldehyde, respectively, and attachment of the carboxyl group to C2 of the indole ring (Figure 1A).[1b,3] Such a reaction is chemically unprecedented, and despite several investigations,[1b,3,7] the mechanism of the carbon-chain recombination process remains unclear. Herein, we report mechanistic investigation of NosL through the use of the substrate analogue 2-methyl-3-(indol-3-yl)propanoic acid (MIPA, **6**; Figure 1B). A series of isotopic labeling studies showed that, surprisingly, the dAdo-radical-mediated hydrogen abstraction occurs at two different sites. Hydrogen abstractions from the methyl group and the Cβ position of MIPA result in the production of MIA and the desaturated product 2-methyl-3-(indol-3-yl)acrylic acid (MIAA, **7**), respectively (Figure 1B), thus highlighting the remarkable flexibility of NosL catalysis. We also identified an α,β-unsaturated ketone shunt product (1-(3-methylindole-2-yl)propenone, **8**) in the assay mixture (Figure 1B), which provides mechanistic insight into this intriguing biotransformation.

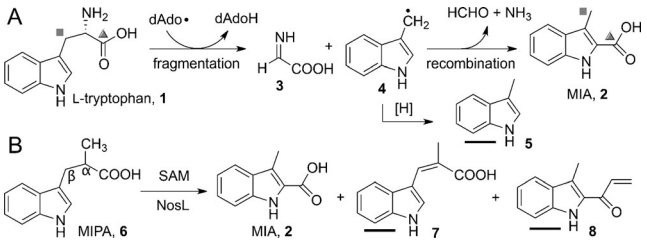

Figure 1. NosL-catalyzed reactions. A) MIA biosynthesis involves an unusual fragmentation–recombination process. The grey rectangles and triangles indicate previous ^{13}C labeling studies of MIA biosynthesis.[1b] B) NosL-catalyzed reaction with the substrate analogue MIPA (**6**), which produced MIA (**2**), desaturated product **7**, and α,β-unsaturated ketone product **8**. The shunt products are indicated by a black line under the structure.

Radical SAM superfamily enzymes utilize a [4Fe-4S] cluster to bind SAM and reductively cleave its carbon–sulfur bond to produce the dAdo radical, which initiates a highly diverse array of biotransformations that are relevant to nucleic acid and protein modification, and the biosynthesis of vitamins, cofactors, and natural products.[8] Unlike most radical SAM enzymes, which initiate the reaction by abstraction of hydrogen atoms from sp^3 carbons, it has been shown that the dAdo radical produced by NosL abstracts a solvent-exchangeable hydrogen in the catalysis;[3] similar observations have also been reported for HydG[4b] and CofH.[6b] By using substrate analogues, we and Begley et al. have recently shown that the dAdo radical does not abstract a hydrogen

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atom from the indole nitrogen of L-tryptophan.[3] Although it is most likely that the dAdo-radical-mediated hydrogen abstraction in NosL catalysis is from the amino group of L-tryptophan, as proposed according to the NosL crystal structure,[7] it is also possible that the dAdo radical initiates the reaction by abstracting a hydrogen atom from a protein residue, as found in the catalysis of the C-P lyase protein PhnJ[9] and the RNA methylases RlmN and Cfr.[10]

To unequivocally establish the dAdo-radical-mediated hydrogen abstraction site in NosL catalysis, we synthesized MIPA (**6**), a tryptophan analogue in which a methyl group replaces the amino group (Figure 1B). Liquid chromatography with high-resolution mass spectrometry (LC–HRMS) analysis of the reaction mixture containing reconstituted NosL, SAM, sodium dithionite, and MIPA (**6**) showed that MIA (**2**) was produced in the assay ($[M+H]^+$ calc. 176.0712, obs. 176.0710, 1.1 ppm error; Figure 2A), albeit with a significantly reduced yield (10–20%) compared to that produced from L-tryptophan, thus suggesting that **6** is a NosL substrate. We then ran the reaction with **6** in a buffer containing 80% D_2O, and LC–MS analysis of the assay mixture showed that deuterium incorporation into 5′-deoxyadenosine (dAdoH) was not apparent (Figure 2B). This is in stark contrast to the observation of multiply deuterated dAdoH produced in a similar reaction with L-tryptophan (Figure 2C). The distinct isotope distributions of dAdoH from the two reactions exclude the possibility that the dAdo radical abstracts a hydrogen atom from a protein residue, because otherwise the dAdoH produced in different reactions should have a similar isotope distribution. To further establish the hydrogen abstraction site in NosL catalysis, we synthesized the deuterated MIPA isotopologue [methyl-$^2H_3$]-MIPA (**6Dm**; Figure 2D). When **6Dm** was used as the substrate, deuterium incorporation into dAdoH was observed (Figure 2E). Combined with previous structural and biochemical studies,[3,7] these analyses unambiguously demonstrate that the dAdo-radical-mediated hydrogen abstraction in NosL catalysis is from the amino group of L-tryptophan and not from a protein residue.

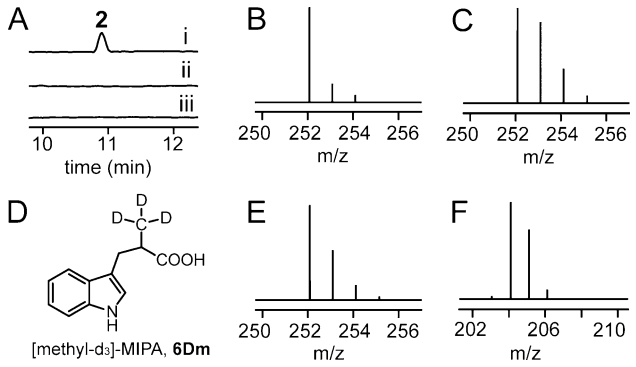

Figure 2. Mechanistic investigation of NosL catalysis by using MIPA as a molecular probe. A) LC–MS analysis of the MIPA reaction mixture, showing extracted ion chromatograms (EICs) for $[M+H]^+ = 176.1$ (corresponding to MIA, **2**) for the NosL-catalyzed reaction with all the required components (i), a control reaction with boiled NosL(ii), and a control reaction in which SAM was omitted (iii). B) Summed MS spectrum of dAdoH produced in the reaction with MIPA in 80% D_2O. C) Summed MS spectrum of dAdoH produced in the reaction with L-tryptophan in 80% D_2O. D) The chemical structure of [Methyl-$^2H_3$]-MIPA (**6Dm**), which was synthesized according to a procedure described in the Supporting Information. E) Summed MS spectrum of dAdoH in the reaction with **6Dm** in H_2O. F) Summed MS spectrum of MIPA (**6**) in the reaction in 80% D_2O.

We also observed apparent deuterium incorporation into the substrate when the reaction was performed in D_2O buffer (Figure 2F). A similar observation has also been reported in the study of the radical SAM enzyme DesII.[11] Surprisingly, when the singly deuterated MIPA produced in the reaction was subjected to HRMS/MS analysis, the result showed that a significant fraction of MIPA was deuterated at the β position (Figure 3A). This suggests that a radical intermediate (**9**; Figure 3B) was produced in the reaction, which can be reduced to [β-^{2}H]-MIPA (**6Dβ**; Figure 3A) by a solvent-derived hydrogen-atom equivalent. To validate this proposal, we synthesized **6Dβ** and incubated it with NosL and other reaction components. LC–HRMS showed that deuterium was indeed (albeit not very efficiently) incorporated into dAdoH in this analysis (Figure 3C), thus suggesting that the dAdo radical can abstract a hydrogen atom from the MIPA Cβ position. The inefficient deuterium incorporation into dAdoH is likely the result of an isotope effect and the fact that **6Dβ** is only singly deuterated at the Cβ position.

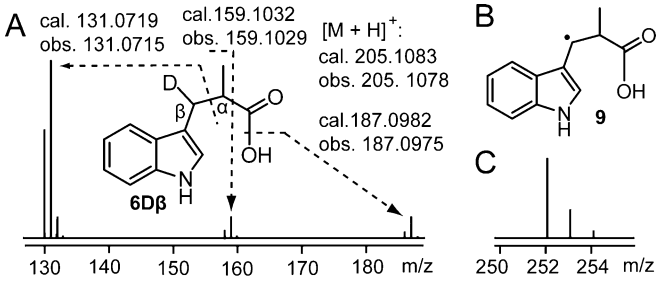

Figure 3. Hydrogen abstraction from the MIPA Cβ position in NosL catalysis. A) HRMS/MS analysis of **6Dβ** produced in the NosL reaction in 80% D_2O, which contains a deuterium atom at the Cβ position. B) A putative radical intermediate (**9**) in the NosL reaction with MIPA. C) Summed MS spectrum of dAdoH in the reaction with **6Dβ** in H_2O.

The identification of **6Dβ** inspired us to perform an extensive survey for all possible products in the reaction mixture. This revealed a product exhibiting a protonated molecular ion at $m/z = 202.0862$, which is absent in the control reaction (Figure 4A, traces i, ii). The suggested molecular formula of this product is $C_{12}H_{11}NO_2$ (2.9 ppm error), which is consistent with the desaturated product MIAA (**7**; Figure 1B). The identity of **7** was further confirmed by co-elution with the synthetic standard in HPLC analysis (Figure 4B, trace iii). We further identified **10** (Figure 4C,D), a product resulting from the Michael addition of dithiothreitol (DTT) to **7**, and the identity of **10** is supported by HRMS/MS analysis (Figure S1). Notably, when **6Dm** was reacted with NosL, a product that is consistent with [methyl-$^2H_3$]-MIAA (**7D**; Figure 4E) was observed ($[M+H]^+$ cal. 205.1056, obs. 205.1045, 5.3 ppm error). This product contains an intact

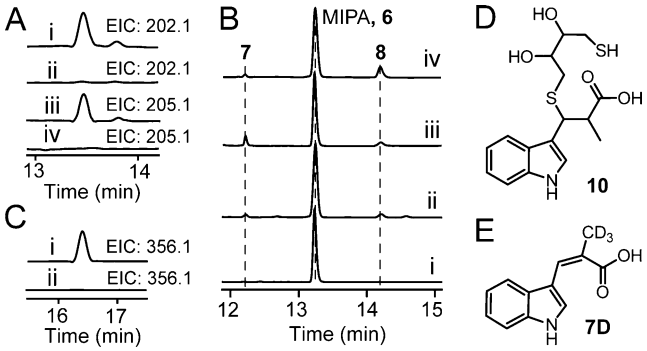

Figure 4. Identification of the shunt products in the NosL-catalyzed reaction with MIPA. A) LC–MS analysis of the assay mixture, showing the extracted ion chromatograms (EICs) of $[M+H]^+ = 202.1$ (corresponding to **7**) for the NosL reaction (i) and a control reaction in which the supernatant of boiled NosL was used (ii); and the EIC of $[M+H]^+ = 205.1$ (corresponding to **7D**) for the NosL reaction with **6Dm** (iii) and a negative control reaction (iv). B) HPLC analysis of the MIPA reaction, using the supernatant of boiled NosL as a negative control (i) or reconstituted NosL as the catalyst (ii); the reaction mixture was also co-injected with synthetic **7** (iii) or the synthetic **8** (iv) to confirm the production of **7** and **8** in the reaction. C) The extracted ion chromatograms (EICs) of $[M+H]^+ = 356.1$ (corresponding to **10**) for the NosL reaction (i) and a negative control reaction (ii). D) The structure of **10**, a DTT adduct of **7**. E) The structure of [methyl-$^2H_3$]-MIAA (**7D**), which was produced in the NosL reaction with **6Dm** as the substrate.

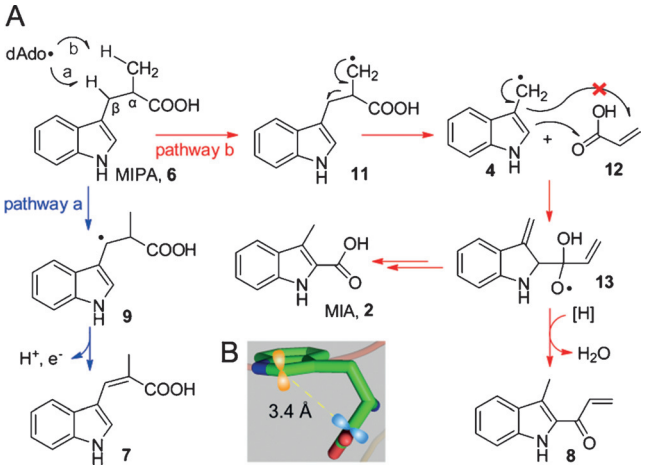

Figure 5. Tuning NosL catalysis with MIPA. A) Proposed mechanism for the NosL-catalyzed production of **2**, **7**, and **8**. B) The bent conformation of L-tryptophan in the NosL crystal structure (PDB ID: 4R33).

triply deuterated methyl group, thus suggesting that **7** likely originates from the radical intermediate **9** (Figure 3B). We also observed the production of a compound that exhibited a protonated molecular ion at $m/z = 186.0917$ in the assay mixture. The suggested molecular formula of the product is $C_{12}H_{11}NO$ ($[M+H]^+$ calc. 186.0919, 1.1 ppm error), which is consistent with the α,β-unsaturated ketone product **8** (Figure 1B). Indeed, the fragmented ions of this compound at $m/z = 158.06$ and 130.07 in HRMS/MS analysis matched well with the proposed structure (Figure S2), and the identity of **8** was further confirmed by co-elution with the synthetic standard in HPLC analysis (Figure 4B, trace iv).

The result presented above demonstrate that MIPA is partitioned into two parallel pathways through two types of dAdo-radical-mediated hydrogen abstraction in NosL catalysis (Figure 5A). Hydrogen abstraction from Cβ (pathway a) results in the production of radical intermediate **9**, which can be further oxidized to generate the desaturated product **7**. Alternatively, the dAdo radical can abstract a hydrogen atom from the methyl group (pathway b), resulting in cleavage of the Cα–Cβ bond of **6** and production of the indolyl radical **4** and acrylic acid (**12**). Radical **4** then attacks the carboxyl group of **12** to produce the α-hydroxyalkyloxy radical **13** (Figure 5A), and this highly unusual regioselectivity is apparently determined by the geometry of the enzyme active site and the spatial orientation of the substrate and reaction intermediates. Consistently, in the NosL crystal structure, L-tryptophan adopts a bent conformation and the distance between the indole C2 and the carboxyl carbon atom is only 3.4 Å (Figure 5B),[7] which is only slightly larger than the sum of the van der Waals radii of the two carbon atoms (ca. 3.0 Å, assuming 1.5 Å for the van der Waals radius of an sp^2 carbon atom).[12] The α-hydroxyalkyloxy radical **13** can then undergo a C–C bond scission to produce the final product MIA (**2**). Alternatively, **13** can be reduced and converted into **8**. According to this analysis, **7** is not an intermediate but a shunt product in the reaction. To confirm this proposal, we ran the reaction in a buffer containing 67% $H_2{}^{18}O$. LC–MS analysis of the reaction mixture showed that neither **8** nor **2** underwent apparent ^{18}O enrichment (Figure S3), thus indicating that the carboxyl group of L-tryptophan remains intact in **2**. Similarly, **2** showed no apparent ^{18}O enrichment when L-tryptophan was used as the substrate (Figure S4).

In summary, through the use the substrate analogue MIPA (**6**) and identification of the shunt products in the assay mixture, this investigation highlights the remarkable catalytic flexibility of NosL and supports a biochemically unprecedented radical addition process by which the carboxyl group is installed on C2 of the indole ring. The unusual regioselectivity is likely achieved by precise tuning of the reactivity of the radical intermediates, and such a highly controlled radical catalysis is likely ubiquitous in the radical SAM superfamily, as proposed in the study of 2,3-lysine aminomutase.[13] Small perturbation in the delicate controlling system within the enzyme active site could therefore significantly change the catalytic outcome, and this has been clearly demonstrated in recent studies on NosL with unnatural substrates and mutagenesis.[3]

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