# Expanding Radical SAM Chemistry by Using Radical Addition Reactions and SAM Analogues

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Abstract: *Radical S-adenosyl-ʟ-methionine (SAM) enzymes utilize a [4Fe-4S] cluster to bind SAM and reductively cleave its carbon–sulfur bond to produce a highly reactive 5′-deoxyadenosyl (dAdo) radical. In almost all cases, the dAdo radical abstracts a hydrogen atom from the substrates or from enzymes, thereby initiating a highly diverse array of reactions. Herein, we report a change of the dAdo radical-based chemistry from hydrogen abstraction to radical addition in the reaction of the radical SAM enzyme NosL. This change was achieved by using a substrate analogue containing an olefin moiety. We also showed that two SAM analogues containing different nucleoside functionalities initiate the radical-based reactions with high efficiencies. The radical adduct with the olefin produced in the reaction was found to undergo two divergent reactions, and the mechanistic insights into this process were investigated in detail. Our study demonstrates a promising strategy in expanding radical SAM chemistry, providing an effective way to access nucleoside-containing compounds by using radical SAM-dependent reactions.*

The radical SAM superfamily represents the largest known enzyme superfamily, consisting of more than 165 000 members found in all three domains of life.[1] These enzymes utilize a [4Fe-4S] cluster to bind S-adenosyl-ʟ-methionine (SAM) in a bidentate fashion and reductively cleave its carbon-sulfur bond to produce a highly reactive 5′-deoxyadenosyl (dAdo) radical.[1b–e,2] Radical chemistry is then imposed on a variety of organic substrates, leading to a highly diverse array of biotransformations.[3] The dAdo radical can also be produced from adenosylcobalamin via C—Co bond homolysis by the B12 radical superfamily enzymes.[4] For both radical SAM and B12 radical enzymes, the job of the dAdo radical is to abstract a hydrogen atom from substrates or enzymes to initiate the reaction. To date, the menaquinone biosynthesis enzyme MqnE represents the only known radical SAM member that

has evolved to catalyze the addition of a dAdo radical to an olefin (Figure 1 A).[5] The rarity of the dAdo-radical-based addition in radical SAM superfamily enzymes is in stark contrast to the prevalence of radical addition reactions in organic chemistry.[6] Herein, we report the change of the
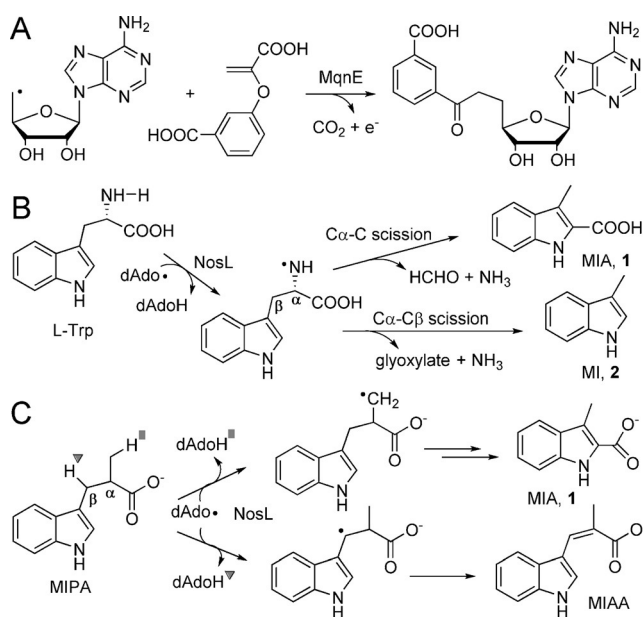
Figure 1. Reactions catalyzed by the radical SAM enzymes MqnE and NosL. A) The MqnE-catalyzed dAdo radical-based addition reaction involved in menaquinone biosynthesis. B) The NosL-catalyzed promiscuous reactions with the authentic substrate ʟ-Trp. C) Two routes of hydrogen abstraction in NosL reaction with an unnatural substrate MIPA. The two hydrogens abstracted by the dAdo radical are shown by triangles and rectangles, respectively.

dAdo-radical-based reaction of the enzyme NosL from hydrogen abstraction to radical addition by using a double-bond-containing substrate analogue. We show that NosL accepts different SAM analogues with varied base moieties, allowing effective production of different nucleoside adducts.

NosL is a radical SAM enzyme that catalyzes the carbon-chain rearrangement of ʟ-tryptophan (ʟ-Trp) to produce 3-methyl-2-indolic acid (MIA, **1**; Figure 1 B), a key intermediate in the biosynthesis of the clinically interesting thiopeptide antibiotic nosiheptide.[7] This remarkable carbon-chain rearrangement reaction was recently suggested to proceed via fragmentation of the ʟ-Trp Cα—C bond and subsequent migration of the carboxyl-fragment radical to the indole C2 (Figure 1 B).[8] NosL also catalyzes the scission of the ʟ-Trp Cα—Cβ bond to produce 3-methylindole (MI, **2**) and glyox-

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ylate (Figure 1 B).[7b] Both Cα−C and Cα−Cβ fragmentations of L-Trp result from the dAdo radical-mediated hydrogen abstraction from the L-Trp amino group (Figure 1 B).[9] We recently showed that NosL is also able to produce MIA from 2-methyl-3-(indolyl)propanoic acid (MIPA), a Trp analogue in which a methyl group replaces the amino group, and this transformation is initiated by hydrogen abstraction from the methyl group of MIPA (Figure 1 C).[10] NosL also abstracts a hydrogen from the MIPA Cβ and produces a desaturated product 2-methyl-3-(indolyl)acrylic acid (MIAA; Figure 1 C), demonstrating the remarkable catalytic promiscuity of this enzyme.[10] Based on these observations, we reason that NosL is likely able to recognize a MIPA structural analogue that contains an olefin moiety, and in this case, the dAdo radical would add to the olefin instead of initiation of the canonical hydrogen abstraction process.

To test this hypothesis, we synthesized 2-(indolylmethyl)-acrylic acid (IMAA, **3**) and used it in a NosL assay (Figure 2 A). Incubation of IMAA with SAM, sodium dithionite, and the reconstituted NosL resulted in a product with a protonated molecular ion at $m/z = 453.1883$ in liquid
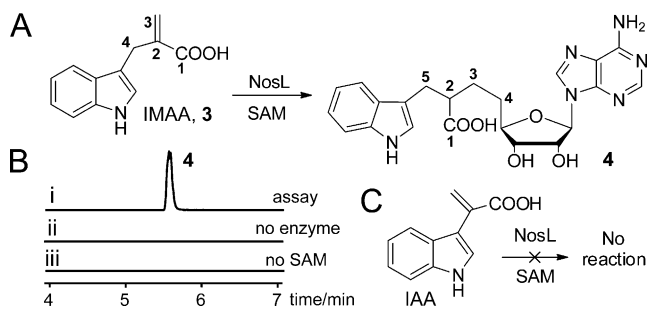
Figure 2. Investigation of NosL reaction by using L-Trp analogues. A) NosL-catalyzed reaction with IMAA **3**, which produces an adenosine adduct **4**. B) LC-MS analysis of the IMAA reaction mixture, showing the extracted ion chromatograms (EICs) of $[M+H]^+ = 453.2$ (corresponding to **4**) for i) NosL-catalyzed reaction with all the required components, ii) control reaction with boiled NosL, and iii) control reaction in which SAM was omitted. C) NosL does not act an IMAA analogue 2-(indolyl)acrylic acid (IAA).

chromatography (LC)/high-resolution mass spectrometry (HR-MS) analysis (Figure 2 B). The suggested molecule formula $C_{22}H_{24}N_6O_5$ ($[M+H]^+$ calc. 453.1886, 0.7 ppm error) is consistent with an adenosine adduct of IMAA **4** (Figure 2 A), and this is supported by detailed HR-MS/MS analysis (Figure S1). In contrast to the low production yield of MIA (**1**), production of **4** is efficient, with a yield more than 10-fold higher than that of MIA (Figure 3 A). The relatively high yield of **4** allowed recovering a sufficient amount of the compound for NMR analysis (Figures S2–S3), which unequivocally showed that the dAdo radical-based addition proceeds regiospecifically onto the C3, not C2, of IMAA (Figure 2 A). HPLC analysis using a prolonged elution program showed that **4** likely consists of two diastereoisomers, and one diastereoisomer (which likely has a *2R*-configuration, see below) accounts for approximately 75 % of the total amount (Figure S4). Production of 5′-deoxyadenosine (dAdoH), the
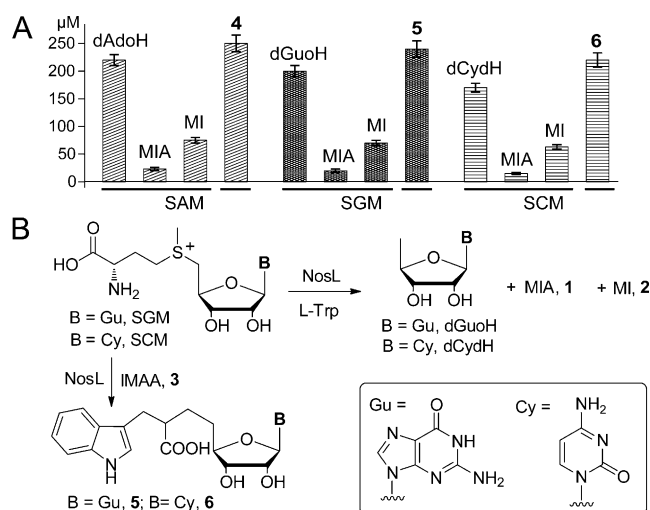
Figure 3. NosL acts on two SAM analogues SGM and SCM. A) The yields of different products in NosL reaction with SAM, SGM, or SCM. Assays were carried out by incubating 500 μM substrates (L-Trp or IMAA, **3**) with 50 μM reconstituted protein, 500 μM SAM, and 2 mM of sodium dithionite in 50 mM MOPS buffer (pH 8.0) for 1.5 h. Assays were performed in duplicates, and the standard deviations (S.D.) are shown by the error bars. B) Two types of reactions (hydrogen abstraction and radical addition) catalyzed by NosL with the SAM analogues.

characteristic product of the radical SAM superfamily enzymes, was barely observable in the reaction (Figure S5), suggesting that the dAdo radical was almost exclusively trapped by IMAA rather than being reduced to dAdoH. Despite the remarkable catalytic promiscuity of NosL-catalyzed hydrogen abstraction reactions (Figure 1 C),[10,11] our analysis showed that NosL did not act on 2-(indolyl)-acrylic acid (IAA), an IMAA analogue lacking a methylene group of IMAA (Figure 2 C).

Unlike typical radical SAM chemistry, in which dAdo radical only serves as a radical initiator, the dAdo-radical-based addition reaction allows efficient adding of a nucleoside moiety to the substrate. Production of the adenosine adduct **4** by NosL thus raises the tempting possibility of diversifying radical SAM-dependent reactions by using SAM analogues with different nucleotide functionalities. To test this hypothesis, we synthesized *S*-guanosylmethionine (SGM), a SAM structural analogue that has a guanine moiety (instead of an adenine in SAM; Figure 3 B). LC-HR-MS analysis of the reaction mixture containing SGM, L-Trp, sodium dithionite, and the reconstituted NosL showed that production of 5′-deoxyguanosine (dGuoH), the cleavage product of SGM, was indeed observed ($[M+H]^+$ calc. 268.1046, obs. 268.1045, 0.4 ppm error). MIA (**1**) and MI (**2**) were also produced in the reaction, and the yields of dGuoH, MIA, and MI were comparable to those in the reaction with SAM (Figure 3 A), suggesting that SGM is a competent substrate for radical initiation in NosL catalysis. Consistent with this observation, incubation of SGM, IMAA, dithionite, and the reconstituted NosL resulted in a guanosine adduct **5** (Figure 3 B, $[M+H]^+$ calc. 469.1836, obs. 469.1831, 1.1 ppm error), and the structure is supported by comparative HR-MS/MS analysis (Figure S6). These results demonstrate the possibility of expanding radical

SAM-dependent reactions by employing radical addition reactions with a SAM analogue.

To further explore the substrate tolerance of NosL, we synthesized another SAM analogue, *S*-cytidinylmethionine (SCM), which contains a pyrimidine (instead of a purine in SAM), therefore presenting a much bigger structural variation from SAM compared to SGM (Figure 3B). To test whether SCM is able to initiate NosL reaction, SCM was incubated in an assay mixture containing L-Trp, dithionite, and the reconstituted NosL. HPLC and LC-HR-MS analysis showed that MIA (**1**), MI (**2**), and 5′-deoxycytidine (dCydH) were all produced in the assay, and the yields are slightly decreased but comparable to those in the reaction with SAM (Figure 3A). These results suggest NosL is able to effectively cleave SCM and use a 5′-deoxycytidinyl radical to initiate the reaction. Consistent with these results, a cytidine adduct **6** was produced in the assay mixture containing SCM, IMAA, and other required components (Figures 3A and S7). The competence of SGM and SCM in the NosL reaction suggests that, although conserved interactions are found in radical SAM enzymes for binding of the adenine moiety of SAM,[2a] these interactions are possibly not indispensable for SAM recognition.

Unlike the reaction catalyzed by MqnE, in which the dAdo radical adduct loses an electron and is decarboxylated after the radical-mediated intermolecular rearrangement (Figure 1A), the dAdo radical adduct with IMAA in NosL reaction is reduced by a hydrogen equivalent. To reveal the source of this hydrogen atom, we performed the assay with IMAA in D_2O buffer (pD 8.0, 90% D_2O). LC-HR-MS analysis of the resulting assay mixture showed that **4** is predominately mono-deuterated (Figure 4A), suggesting that the radical intermediate **7** is mainly quenched by a solvent-exchangeable hydrogen equivalent, likely through hydrogen abstraction from a solvent-exchangeable site (Figure S8). Molecular docking analysis showed that the hydroxyl group of NosL Tyr90 is close to the IMAA C2 (Figure S9A), which could possibly provide a hydrogen source for **7** reduction. The fact that Tyr90 resides at the *Si* face of IMAA also suggests

that the majority of **4** likely has a (2*R*)-configuration (Figure S9B). Notably, a small proportion ($\approx 12\%$) of di-deuterated **4** was observed in the assay mixture (Figure 4A). A possible explanation is that the hydrogen abstraction in **4** production is a reversible event, which can also occur on other sites of **4** (for example, the C5 of **4**; Figure S8). Indeed, detailed HR-MS/MS analysis of the di-deuterated **4** showed that the two deuterium atoms are on the IMAA-derived moiety, and part of the C5 of **4** was deuterated (Figure S10). These results further highlighted the remarkable conformational diversity of NosL and the promiscuous substrate binding in the enzyme active site.[10b]

Interestingly, we also observed a product that exhibits a protonated molecular ion at $m/z = 605.1848$ in LC-HR-MS analysis (Figure S11). Detailed HR-MS/MS analysis showed an apparent neutral loss of $m/z = 120. 02$ ($m/z = 470.13$, and 350.11; Figures 4B and S11B) that is reminiscent of a dithiothreitol (DTT) adduct observed in our previous study.[10a] Indeed, the suggested molecule formula of $C_{26}H_{32}N_6O_7S_2$ ($[M+H]^+$ calc. 605.1852, 0.7 ppm error) is consistent with **9**, a structural analogue of **4** containing an extra DTT moiety (Figure 4B). Observation of **9** suggested that NosL produced a desaturated product **8** in the assay (Figure 4B). Indeed, examination of the LC-HR-MS data clearly showed that **8** was produced in the assay mixture, which exhibits a protonated molecular ion consistent with the predicted molecular formula $C_{22}H_{22}N_6O_5$ ($[M+H]^+$ calc. 451.1730, obs. 451.1726, 0.9 ppm error; Figure S12). Production of the desaturated product **8** suggested that besides being reduced by a solvent-exchangeable hydrogen equivalent, the radical adduct **7** can also undergo a one-electron-oxidation coupled with deprotonation of the C5 methylene group. The regio-specificity of this desaturation process is likely a result of the relatively higher activity of the C5 hydrogen compared to that of C3 (Figure 4B). The analogues of **8** and **9** that contain guanine or cytosine were also observed in the reactions when SGM or SCM were used (Figure S13).

We next performed the reaction with different concentration of sodium dithionite. HPLC analysis of the resulting assays showed that the yield of **4** increased with the increased dithionite concentration (Figure S14), supporting the requirement of external electron donor to facilitate **4** production. However, the production of **8** and **9** remains constantly low in all of the dithionite concentrations, and was not observed when the dithionite concentration was very high (> 5 mM). To further interrogate the redox stoichiometry in **8** formation, reconstituted NosL was incubated overnight with sodium dithionite, allowing the [4Fe-4S] cluster to be fully reduced and the excess dithionite to decompose via disproportionation, and the reduced enzyme was subsequently incubated with SAM and IMAA in the absence of dithionite. This redox stoichiometry analysis was developed by Liu and co-workers in the study of DesII,[12] and was recently used in our lab in studying the radical SAM-dependent amine dehydrogenation reactions.[11] The UV/Vis spectrum of the reconstituted NosL upon overnight reduction is similar to that of the freshly reduced enzyme (Figure S15), supporting that the pre-reduced enzyme was functional. LC-HR-MS analysis of the reaction with the pre-reduced enzyme in the absence of
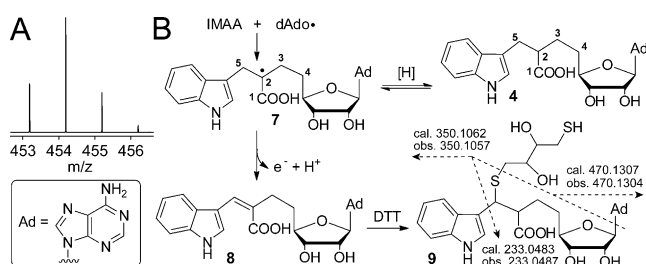
Figure 4. Mechanistic insights into the NosL-catalyzed dAdo radical-based addition reaction. A) The mass spectrum of **4** produced in the NosL reaction with IMAA in D_2O buffer (pD 8.0, 90% D_2O). The spectrum clearly shows that **4** is mainly mono-deuterated and partially di-deuterated. B) Divergence of the radical intermediate **7** into two reaction pathways, producing the reduced product **4** and the desaturated product **8**, the latter is further converted to a DTT adduct **9** by Michael addition of DTT to **8**. Part of the collision-induced dissociation (CID) ion fragments of **9** was shown, and the full HR-MS/MS spectrum of **9** and **8** were shown in Figures S11B and S12B, respectively.

external reductant showed that **4**, **8**, and **9** were all produced, and the total amount of the three products is at substoichiometric levels of enzyme (Figure S16). These results indicated that, unlike the NosL-catalyzed amine dehydrogenation reactions,[11] one-electron oxidation of the radical intermediate **7** cannot be coupled with regeneration of the reduced [4Fe-4S] cluster (Figure 4B), and the electron acceptor in **8** production is unclear at this stage.

In summary, we successfully transformed the dAdo radical-based chemistry from hydrogen abstraction to radical addition in NosL catalysis by using an olefin-containing substrate analogue. Such a strategy could be well applied to other radical SAM enzymes, as has also been shown in an early report on pyruvate–formate lyase (PFL) activating enzyme.[13] We showed that two SAM analogues with different nucleoside functionalities are recognized by the enzyme, which initiate the radical-based reactions with high efficiencies comparable to that by SAM. Combination of radical addition chemistry and use of SAM analogues could significantly expand the current repertoire of the radical SAM enzymology, providing an effective way to access nucleoside-containing compounds, which represent a promising source of leads in pharmaceutical research.[14] Our study also complements the potentially diverse role of dAdo radical in radical SAM chemistry, as was recently highlighted by the observation of a transient organometallic adenosyl species in the catalysis of PFL activating enzyme,[15] and by the unique thioether chemistry in the reaction catalyzed by the hydrogenase-maturing enzyme HydE.[16]

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