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The Catalytic Mechanism of the Class C radical *S*-Adenosylmethionine Methyltransferase NosN

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Abstract: *S-Adenosylmethionine (SAM) is one of the most common co-substrates in enzyme-catalyzed methylation reactions. Most SAM-dependent reactions proceed through an S_N2 mechanism, whereas a subset of them involves radical intermediates for methylating non-nucleophilic substrates. Herein, we report the characterization and mechanistic investigation of NosN, a class C radical SAM methyltransferase involved in the biosynthesis of the thiopeptide antibiotic nosiheptide. We show that, in contrast to all known SAM-dependent methyltransferases, NosN does not produce S-adenosylhomocysteine (SAH) as a co-product. Instead, NosN converts SAM into 5′-methylthioadenosine as a direct methyl donor, employing a radical-based mechanism for methylation and releasing 5′-thioadenosine as a co-product. A series of biochemical and computational studies allowed us to propose a comprehensive mechanism for NosN catalysis, which represents a new paradigm for enzyme-catalyzed methylation reactions.*

Methyltransferases are prevalent in biology and are essential for almost all life processes. The vast majority of methyltransferases use *S*-adenosylmethionine (SAM) as the methyl donor, and typically the methyl group is transferred from SAM to a nucleophile through an S_N2 mechanism.[1] Methylation can also occur at non-nucleophilic centers such as inert carbon or phosphorous atoms through radical-based mechanisms.[2] Currently, all of the known radical-based methyltransferases belong to the radical SAM superfamily, a large enzyme superfamily consisting of more than 16,500 members found in all three domains of life.[3] Radical 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, which initiates a highly diverse array of reactions, including the methylation of various non-nucleophilic substrates.[2] While most radical SAM methyltransferases (RSMTs) share a similar strategy in using SAM both as a methyl donor and a radical initiator, the catalytic mechanisms of RSMTs are diverse.[2]

NosN is a radical SAM enzyme that is involved in the biosynthesis of nosiheptide (**1**),[4] a clinically interesting thiopeptide antibiotic produced by *Streptomyces actuosus* (Figure 1 A).[5] Nosiheptide belongs to the large class of ribosomally synthesized and posttranslationally modified peptides (RiPPs).[6] It contains a precursor-peptide-derived macrocyclic ring that consists of a central tetrasubstituted pyridine and five thiazoles, and a ribosomally-independent side ring system containing an indolic acid moiety (Figure 1 A). Previous study have shown that the *nosN*-knockout mutant of *S. actuosus* does not produce nosiheptide but rather the nosiheptide analogue **2**, in which the indolic side ring system is incomplete (Figure 1 A), thus suggesting that NosN

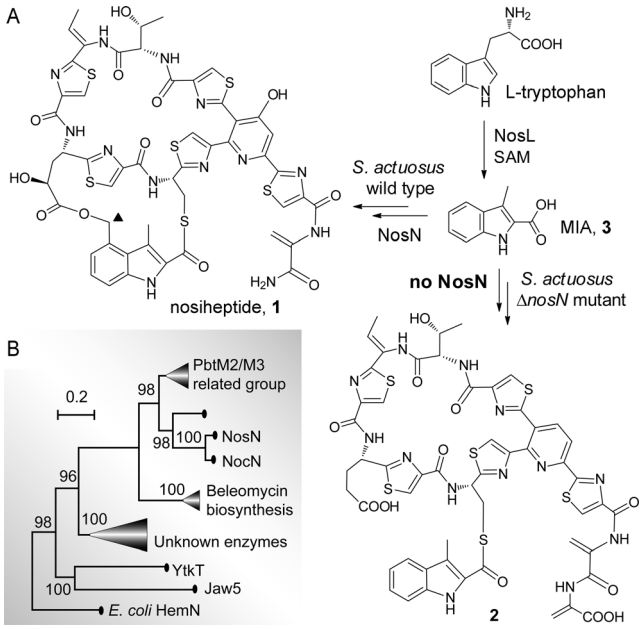

Figure 1. Functional diversity of the class C RSMTs. A) The biosynthesis of nosiheptide in *S. actuosus* involves the class C RSMT NosN. The carbon atom introduced by NosN is indicated with a solid triangle. The *nosN*-knockout mutant of *S. actuosus* does not produce nosiheptide, but rather the nosiheptide analogue **2** (Figure S1–S3). B) Bayesian MCMC phylogeny of the class C RSMTs and other HemN-like enzymes (for details of the phylogenetic analysis, see Methods in the Supporting Information).

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is likely responsible for appending a methyl group on the C4 of the indolyl moiety during nosiheptide maturation.[4]

The fact that NosN is a methyltransferase is further supported by the observation that this enzyme shares significant sequence similarity with other known methyltransferases, such as PbtM2 and PbtM3, which are involved in GE2270 biosynthesis,[7] and YtkT, which is involved in yatakemycin biosynthesis.[8] NosN, YtkT, PbtM2, PbtM3, and some other putative methyltransferases are homologous to the coproporphyrinogen III oxidase HemN (Figure 1B), and are classified as class C RSMTs.[2a,c] Notably, HemN catalyzes two sequential steps of oxidative decarboxylation of coproporphyrinogen III,[9] a reaction that is totally unrelated to methylation. Although the methyltransferase activity of YtkT has been successfully reconstituted in vitro by Tang and co-workers,[8] and ChuW has very recently been reconstituted by Lanzilotta and co-workers,[10] the catalytic mechanisms of the class C RSMTs remain largely unknown.

To interrogate the function and mechanism of NosN, the *nosN* gene was amplified from the genomic DNA of *S. actuosus* and expressed in *Escherichia coli* with an N-terminal hexa-histidine tag, and the protein was purified by Ni^{2+} affinity chromatography under strictly anaerobic conditions. After chemical reconstitution of the [4Fe-4S] cluster, followed by gel-filtration, the protein was found to contain 4.5 ± 0.2 mol Fe and 4.8 ± 0.3 mol S per mol protein. UV/Vis spectroscopy showed that the protein solution had a broad absorption around 415 nm (Figure S4), a feature characteristic of [4Fe-4S]-containing proteins. Analysis of the reaction mixture containing SAM, sodium dithionite, and the reconstituted NosN showed that 5′-deoxyadenosine (dAdoH) was produced in the assay mixture (Figure 2A, trace ii), thus suggesting that NosN is a radical SAM enzyme.

A possible substrate of NosN is 3-methyl-2-indolic acid (MIA, **3**), a nosiheptide biosynthetic intermediate produced from L-tryptophan by the radical SAM enzyme NosL (Figure 1A).[11] To test whether MIA is the NosN substrate, we performed the NosN assays with MIA and carefully examined the assay mixture by liquid chromatography high resolution mass spectrometry (LC–HRMS). No methylated product was found in the assay mixture, thus suggesting that MIA is probably not the substrate of NosN. Another possible substrate is **2**, which is produced by the *nosN*-knockout mutant (Figure 1A). We obtained the purified compound **2** from the *nosN*-knockout mutant of a nosiheptide high-producing strain (Methods and Figure S1–S3 in the Supporting Information), and performed the assays with **2** and the other required components. Again, no methylated product was observed in the assay mixtures, suggesting that **2** is likely not the NosN substrate but rather an off-pathway product produced by the mutant strain.

Because the indolic acid moiety in nosiheptide is attached to a cysteine thiol as a thioester (Figure 1A), it is possible that MIA is first converted into an activated form before being methylated by NosN and incorporated into the nosiheptide scaffold.[4] Thioester linkage to the cysteamine group of a phosphopantetheinyl cofactor is arguably the most common strategy used by nature for the activation of a carboxylate, and in biochemical studies, *N*-acetylcysteamine (SNAC) thioesters (Figure 2B) have been widely used as surrogates of phosphopantetheinylated substrates.[12] We therefore synthesized SNAC thioester **4** (Figure 2B) and used it as a potential substrate in the NosN assays. In this analysis, a product with a protonated molecular ion at $m/z = 291.1161$ in LC–HRMS analysis was observed (Figure 2C and Figure S5), which is absent in all the three negative control reactions (Figure S6). The suggested molecule formula $C_{15}H_{18}N_2O_2S$ ($[M+H]^+$ calc. 291.1167, 2.0 ppm error) is consistent with the methylated product **5** (Figure 2B), the identity of which was further supported by comparative HRMS/MS analysis (Figure S7). To further confirm the production of **5** in the reaction, we treated the deproteinized fraction of the assay mixture with NaOH, which leads to hydrolysis of any thioesters, and the resulting mixture was subjected to LC–HRMS analysis. As expected, a product consistent with 3,4-dimethyl-2-indolic acid (**6**) was observed ($[M-H]^-$ calc. 188.0711, obs. 188.0710, 0.5 ppm error), and its identity was further supported by comparison with two synthetic dimethylindolic acid isomers (Figure S8). These results clearly indicate that NosN is a novel radical SAM methyltransferase that is responsible for appending a methyl group onto C4 of the indolyl moiety in nosiheptide biosynthesis.

We next synthesized *S*-adenosyl-L-[methyl-$^2H_3$]-methionine (d_3-SAM) and used it in the NosN assay. LC–HRMS

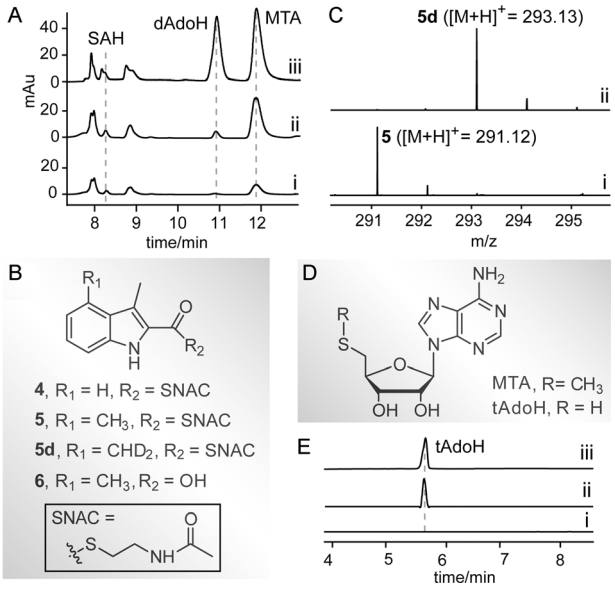

Figure 2. Mechanistic investigation of NosN. A) HPLC analysis of the NosN assay mixture, using a supernatant with boiled NosN as a negative control (i), and reconstituted NosN with the other required components in the absence (ii) or presence (iii) of **4**. Because of the low yield and the similar retention time to that of dAdoH, tAdoH is not visible in this analysis. B) Structures of the indolyl compounds in relation to our biochemical analysis. C) MS spectra of **5** (i) and **5d** (ii) produced in the NosN-catalyzed reaction with SAM or d_3-SAM, respectively. D) Chemical structures of the MTA and tAdoH produced in the NosN reaction. E) LC–MS analysis of NosN reaction mixtures, showing the extracted ion chromatograms (EICs) of $[M+H]^+ = 284.1$ (corresponding to tAdoH) for the control reaction with boiled NosN (i), the NosN reaction (ii), and a synthetic tAdoH standard (iii).

analysis of the resulting mixture showed that the methylated product exhibits a protonated molecular ion at $m/z =$ 293.1282 (3.7 ppm error for a molecular formula of $C_{15}H_{18}D_2N_2O_2S$; Figure 2C), thus suggesting that the product (**5d**) contains a methyl group with two deuterium atoms (Figure 2B). This observation is reminiscent of the reactions catalyzed by the RNA methyltransferases RlmN and Cfr, two class A RSMTs that catalyze reactions involving methylated cysteine residues.[13] To test whether NosN uses a similar catalytic strategy involving a protein-bound methyl group, we performed an assay under single-turnover conditions with a limited amount of d_3-SAM. LC–HRMS analysis of the assay mixture showed that, unlike the single-turnover reactions catalyzed by RlmN and Cfr, in which exogenous SAM is not the source of the appended methyl group,[13b] only **5d** was produced in this assay, and the unlabeled product **5** was not observed. This analysis suggests that NosN is different from RlmN and Cfr and does not use a protein-bound methyl group. To further validate this proposal, Cys 50 and Cys 108, the two Cys residues besides those required for [4Fe-4S] binding, were each replaced by an Ala, and the resulting mutant enzymes were reconstituted and tested in the same assay used for the wild type NosN. The results showed that both mutants are able to produce **5** from **4**, thus further excluding the possibility that NosN employs a methylated Cys residue in catalysis.

It has been shown that in the catalytic reactions of the methylthioltransferases MiaB and RimO, the methyl group is initially attached to the [4Fe-4S] cluster of the enzymes, and exogenous methanethiol can serve as the methyl source in the reaction.[14] However, in an assay with d_3-SAM, NosN, and the other required components in the presence of methanethiol, unlabeled product was not produced in the reaction. This analysis excludes the possibility that NosN uses a strategy similar to MiaB and RimO in placing the methyl group on the [4Fe-4S] cluster before its transfer to the substrate.

We next performed a detailed HPLC analysis to track the fate of SAM in NosN catalysis. We noted that although the reaction produced a substantial amount of dAdoH, the yield of *S*-adenosylhomocysteine (SAH) is low and similar to the levels in negative control reactions where methylation did not occur (Figure 2A), thus suggesting that SAH is likely not a co-product in NosN catalysis. This observation is in contrast to all the SAM-dependent methyltransferases that have been identified thus far, whether they use S_N2 or radical-based mechanisms. Unexpectedly, we found that 5′-methylthioadenosine (MTA) was produced at high yields in the reaction (Figure 2A). Although this compound was also present in the control assay with boiled enzyme, the yields of MTA in the assays with the reconstituted NosN were apparently higher (Figure 2A), thus indicating that MTA could possibly be a relevant intermediate in the catalysis. The yield of MTA in the absence of the reducing system (flavodoxin, flavodoxin reductase, and NADPH) is similar to that for the normal reaction, thus suggesting that MTA production is independent of SAM reductive cleavage.

MTA is a known co-product in the biosynthesis of polyamine, ethylene, and diphthamide,[15] and also a product of SAM degradation.[16] However, to the best of our knowledge, there is no biochemical precedent for the participation of MTA in a methylation reaction. We ran the NosN assay with MTA, d_3-SAM, and the other required components, and both **5** and **5d** were observed in the reaction (Figure S9). This analysis supports the idea that MTA is a direct methyl donor in NosN catalysis, which raises the possibility that 5′-thioadenosine (tAdoH; Figure 2D) is released as a co-product in the reaction. Re-examination of the LC–HRMS data showed that tAdoH was indeed produced in the assay (Figure 2E), and the identity of this compound was supported by coelution with the synthetic standard (Figure 2E) and by comparative HRMS/MS analysis (Figure S10). Time course analysis of the reaction mixture showed that tAdoH and **5** were produced in a roughly 1:1 ratio (Figure S11), thus suggesting that tAdoH is likely an authentic co-product in the NosN reaction.

The results presented above suggest that NosN employs an unprecedented mechanism for indole C4 methylation (Figure 3). During catalysis, the enzyme converts one SAM molecule into MTA as a direct methyl donor and a second SAM into a dAdo radical. The dAdo radical then abstracts a hydrogen atom from the MTA methyl group, and the resulting methylene radical **7** adds to the indolyl substrate at C4 to form key radical intermediate **8**. As in the mechanism proposed for the RlmN-catalyzed reaction,[2c] **8** is likely deprotonated to produce radical anion **9**, which undergoes C–

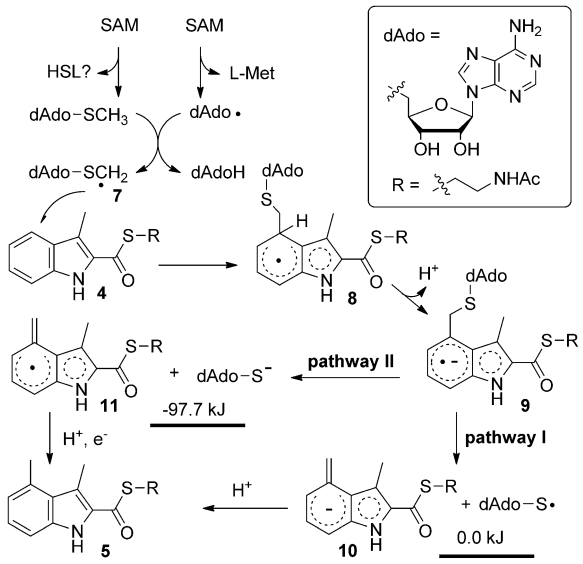

Figure 3. Proposed mechanism for the NosN-catalyzed methylation reaction. In the enzyme active site, one SAM is converted into MTA and the other is reductively cleaved to produce a dAdo radical. The yield of homoserine lactone (HSL), the product of SAM degradation, is low in the reaction, and owing to its lability under the assay conditions, whether it is a co-product or not remains to be further validated. DFT calculations on the model compounds suggests that for the radical anion **9**, heterolytic cleavage of its C–S bond in pathway II is energetically favored over homolytic cleavage (pathway I, the model products of this pathway were used as the reference zero energy). Both geometry optimization and energy were calculated at the B3LYP/6-311+G(2d,p)/SMD(H_2O) level.

S bond cleavage to eliminate the thioadenosine moiety (Figure 3). Density functional theory (DFT) calculations and orbital analysis of the model compound showed that the highest occupied molecular orbital (HOMO α) of **8** has apparent spin density on the C4 hydrogen, while this spin density decreases in the corresponding lowest unoccupied molecular orbital (LUMO β, electron hole; Figure S12). This observation suggests that the C4 hydrogen of **8** could possibly be prone to deprotonation, which is consistent with our proposal (Figure 3).

Cleavage of the C–S bond of **9** could occur either homolytically to eliminate a thiyl radical (Figure 3, pathway I), as has been proposed for RlmN,[2c] or heterolytically to eliminate a thiolate (Figure 3, pathway II). DFT calculations on the two sets of model products showed that the heterolytic products are much lower in energy ($-97.7\ kJ\ mol^{-1}$) than the homolytic product, thus suggesting that the reaction likely proceeds through pathway II to produce indolyl radical **11**, which is subsequently reduced by a hydrogen equivalent to produce the methylated product **5** (Figure 3).

Our study presents a new paradigm for SAM-dependent methyltransferases, in which MTA is used as the direct source of the methyl group. Unlike class A RSMTs, which employ a ping-pong mechanism in which two SAM molecules sequentially bind to the enzyme,[13] NosN likely binds two SAMs simultaneously in the active site, where one SAM is converted into MTA and the second into an dAdo radical to initiate the radical-based reaction. The observation of two SAM molecules in the crystal structure of the NosN homologous enzyme HemN[17] is consistent with this analysis. The novel radical SAM chemistry established herein likely applies to other class C RSMTs, and should facilitate the investigation of other HemN-like enzymes, such as the putative cyclopropane synthase Jaw5 (Figure 2 A).[2c,18] Our study also highlights the remarkable evolutionary and functional adaptability of the HemN-like enzymes and the radical SAM superfamily enzymes as a whole, which could possibly provide rich possibilities for bioengineering studies.[19]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biosynthesis · methyltransferases · natural products · radical SAM enzymes · thiopeptides

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Communications

Biosynthesis

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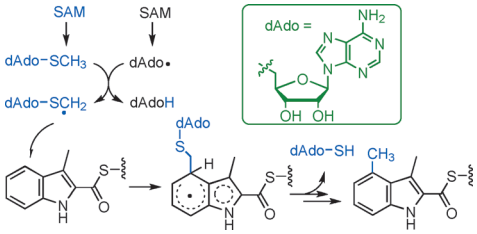
The Catalytic Mechanism of the Class C radical *S*-Adenosylmethionine Methyltransferase NosN



Play something new, SAM: Mechanistic investigation of the class C radical *S*-adenosylmethionine (SAM) methyltransferase NosN showed that, in contrast to all known SAM-dependent methyltransferases, NosN does not produce *S*-adenosylhomocysteine as a co-product. Instead, NosN converts SAM into 5′-methylthioadenosine as a direct methyl donor, employing a radical-based mechanism for methylation and releasing 5′-thioadenosine as a co-product.

