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Radical-mediated enzymatic carbon chain fragmentation-recombination

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The radical *S*-adenosylmethionine (*S*-AdoMet) superfamily contains thousands of proteins that catalyze highly diverse conversions, most of which are poorly understood, owing to a lack of information regarding chemical products and radical-dependent transformations. We here report that NosL, involved in forming the indole side ring of the thiopeptide nosiheptide (NOS), is a radical *S*-AdoMet 3-methyl-2-indolic acid (MIA) synthase. NosL catalyzed an unprecedented carbon chain reconstitution of L-tryptophan to give MIA, showing removal of the Cα-N unit and shift of the carboxylate to the indole ring. Dissection of the enzymatic process upon the identification of products and a putative glycyl intermediate uncovered a radical-mediated, unusual fragmentation-recombination reaction. This finding unveiled a key step in radical *S*-AdoMet enzyme–catalyzed structural rearrangements during complex biotransformations. Additionally, NosL tolerated fluorinated L-tryptophan as the substrate, allowing for production of a regiospecifically halogenated thiopeptide that has not been found among the more than 80 members of the naturally occurring thiopeptide family.

Thiopeptides are a class of clinically interesting and highly modified polythiazolyl antibiotics[1–3]. The potent activity of many members against various drug-resistant bacterial pathogens has promoted extensive investigations by chemical modification into new analog development to overcome their physical drawbacks, such as water solubility, for clinical use. Thiopeptides possess a characteristic macrocyclic core, consisting of a nitrogen-containing 6-membered ring central to multiple thiazoles and dehydroamino acids but vary in side chains (and/or rings), which append additional functionalities (**Fig. 1a**). In contrast to the heroic efforts required for chemical synthesis[4], the newly established biosynthetic pathways for thiopeptide framework formation are remarkably concise[5–11], showing conserved post-translational modifications on a ribosomally synthesized precursor peptide. The reactions include cyclodehydrations and subsequent dehydrogenations to form aromatic thiazoles, dehydrations to generate dehydroamino acids and an intramolecular cyclization to afford the nitrogen heterocycle.

For most polycyclic thiopeptides, the side ring formations are independent of precursor peptide[12–16]. They exclusively exploit L-tryptophan to furnish the variable functional groups, as exemplified by the indolic acid moiety of nosiheptide (NOS) and the quinalic acid moiety of thiostrepton (**Fig. 1b**). Whereas the quinalic acid formation may require seven enzymes to convert L-tryptophan, in a process including methylation, deamination, oxidation, ring opening and recyclization, reduction and epoxidation[6,7], the indolic acid moiety formation in NOS biosynthesis involves the products of two genes, *nosL* and *nosN*, which encode the putative radical *S*-AdoMet proteins that process L-tryptophan via a completely different route[9]. Characterization of a NOS analog that had an open side ring supported the methylation activity of NosN, which acts on the 3-methylindolyl group to furnish the 3,4-dimethylindole moiety. This leaves NosL as a candidate to biosynthesize the key intermediate, 3-methyl-2-indolic acid (MIA, **1**). We have confirmed the essentiality of NosL to **1** formation[9,11]: inactivation of *nosL* completely abolished NOS production, which can be restored either by feeding extraneous **1** into cells or by heterologous complementation with *nosL*'s counterpart *nocL* from nocathiacin (a naturally occurring analog of NOS) biosynthesis. However, whether NosL alone catalyzes such a complex conversion remains unknown, given that **1** biosynthesis from L-tryptophan requires multiple chemically unusual reactions to process the carbon side chain (including removal of the Cα-N unit and shift of the carboxylate to the indole ring; **Fig. 1b**).

In this study, we found that NosL is indeed a radical *S*-AdoMet protein that converts L-tryptophan to **1**, a process that involves a radical-mediated, unusual fragmentation-recombination reaction. Our characterization of NosL took advantage of efficient **1** production in the *nosL*-expressing *Escherichia coli* strain and *in vivo* and/or *in vitro* determination of substrate(s), products (including shunt products) and a putative glycyl intermediate in NosL-catalyzed conversion. In light of the substrate tolerance of NosL, regiospecific fluorination of NOS was achieved via a modified MIA intermediate, yielding a new halogenated thiopeptide that has not yet been found in the family of naturally occurring thiopeptides.

RESULTS

In vivo validation of NosL as a MIA synthase

Considering that radical *S*-AdoMet proteins are sensitive to oxygen in *in vitro* studies[17], we first set out to investigate the NosL function *in vivo* (**Fig. 2**). *nosL* was introduced to and expressed in *E. coli* BL21(DE3), yielding the recombinant strain SL4101 to produce NosL in an N-terminally 6-His–tagged form. HPLC analysis revealed a compound produced by SL4101, which was absent in the control strain SL4100 that carried the vector alone (**Fig. 2a**). The molecular formula for this compound, $C_{10}H_9O_2N$, was established by high-resolution ESI-MS (HR-ESI-MS), showing a $[M - H]^-$ ion at m/z = 174.05605 (174.05615 calculated). Additionally, ^1H, ^{13}C and selected 2D NMR analyses confirmed it to be **1**, indicating that NosL is a new MIA synthase. To probe the origin of **1**, the *nosL*-expressing cells of SL4101 in LB broth were harvested and inoculated into a nutrient-insufficient medium and further incubated with individual amino acids for a time-course analysis of **1** production.

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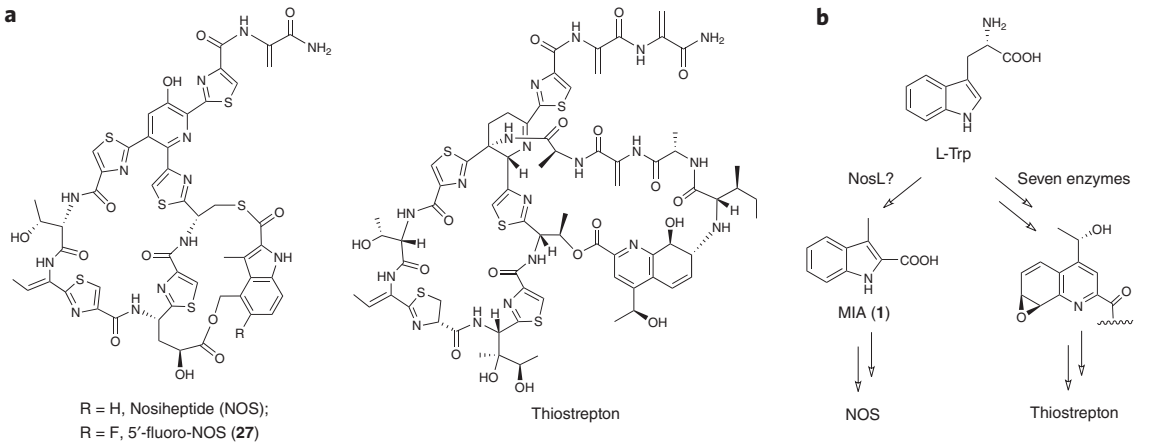


Figure 1 | Structures of polycyclic thiopeptides and their side ring formations. (**a**) NOS, 5′-fluoro-NOS and thiostrepton. (**b**) Processing of L-tryptophan to afford the indolic acid moiety of NOS and the quinalic acid moiety of thiostrepton, respectively, via completely different routes.

Only the addition of L-tryptophan sped up **1** formation with the apparent rate of 150 μg per liter per min in the initial 4 h, whereas supplementation with other amino acids such as L-glycine or L-serine had no substantial effect on the **1**-producing rate (**Supplementary Results** and **Supplementary Fig. 1**). This validated the idea that L-tryptophan serves as the substrate for the production of **1**. The relatively high yield of **1** (42 ± 5 mg per liter over a 20-h period) facilitated its purification and enabled further *in vivo* experiments to examine NosL catalysis.

NosL as a radical *S*-AdoMet protein for MIA formation

On the basis of sequence alignment, NosL falls into the ThiH radical *S*-AdoMet subclass (around 20% identity, **Supplementary Fig. 2**), possessing a typical CxxxCxxC motif for [4Fe-4S] cluster nucleation and a glycine-containing segment involved in *S*-AdoMet binding. We therefore explored the nature of NosL in regard to its cofactor binding and subsequent chemical transformations. In the CxxxCxxC motif, the cysteine residues (corresponding to Cys95, Cys99 and Cys102) were systematically replaced with alanine. The constructs were introduced into *E. coli* BL21(DE3), yielding the recombinant strains SL4103 (for AxxxCxxC mutation of NosL), SL4105 (for CxxxAxxC mutation) and SL4107 (for CxxxCxxA mutation) for **1** examination. These replacements completely abolished **1** production (**Supplementary Fig. 3**), demonstrating their indispensability to NosL activity. The *S*-AdoMet binding potential was also evaluated in a similar way, by replacing Gly142 with alanine within the conserved GE/D motif. The recombinant strain SL4109 that expressed mutant NosL (G142A) lost the ability to produce **1** (**Supplementary Fig. 3**), supporting the idea that NosL is a *S*-AdoMet–dependent protein.

Next, NosL was purified from SL4101 to homogeneity for assays *in vitro*. Although the aerobically isolated enzyme was inactive, the activity of the anaerobically isolated NosL was detectable but extremely low (**Supplementary Fig. 4**). NosL was then reconstituted under strictly anaerobic conditions, giving the purified sample (**Supplementary Fig. 5a**) a dark brownish color. This protein contained 5.1 ± 0.5 of iron and 5.7 ± 0.3 of sulfide per molecule. The UV-visible absorptions of the reconstituted NosL are characteristic for [4Fe-4S] binding proteins (**Supplementary Fig. 5b**), showing a A280/A400 ratio of 3.4:1 and an apparent shift caused by reducing with sodium dithionite. Further electron paramagnetic resonance (EPR) analysis at 13 K gave an axial spectrum (**Supplementary Fig. 5c**) showing approximate *g* values of 2.02 and 1.91. This supported the binding of a $[4Fe\text{-}4S]^{+}$ cluster to NosL. Incubation of the reconstituted NosL with L-tryptophan and *S*-AdoMet was performed in the presence of dithionite, indeed showing **1** production with the yield approximately sevenfold higher than that of the isolated enzyme without reconstitution (**Fig. 2a** and **Supplementary Fig. 4a**). In the reaction mixture the chemical reductant dithionite can be replaced with a natural reduction system containing flavodoxin, flavodoxin reductase and NADPH, leading to ~100% improvement in **1** formation (**Supplementary Fig. 6**). NosL-catalyzed conversion cannot proceed in the absence of *S*-AdoMet (**Fig. 2a**), whose turnover by NosL was further confirmed upon identification of the product 5′-deoxyadenosine (AdoH, **2**, **Fig. 2b**). *S*-AdoMet consumption was independent of L-tryptophan but was significantly enhanced by the addition of L-tryptophan into the reaction mixture (**Fig. 2b** and **Supplementary Fig. 4a**). Together with the identification of methionine (discussed below) as the concomitant product originating from *S*-AdoMet, we conclude that NosL, whose activity is *S*-AdoMet dependent, catalyzes **1** formation from L-tryptophan via a 5′-deoxyadenosyl radical (Ado•)-initiated process.

NosL-catalyzed carbon chain reconstitution on L-tryptophan

To investigate the mechanism of NosL-catalyzed conversion, we focused on the reconstitution of the carbon side chain of L-tryptophan. [1-^{13}C] and [3-^{13}C]-labeled L-tryptophans were fed individually into SL4101, resulting in enriched ^{13}C resonances for the 2-carboxylate (at δ 164.0 for product **3**) and 3-methyl group (at δ 8.83 for product **4**) of MIA, respectively (**Scheme 1** and **Supplementary Fig. 7**). Consistent with similar studies carried out in the NOS-producing strain *Streptomyces actuosus*[12,13], these findings demonstrated that **1** biosynthesis involves an unprecedented carbon chain rearrangement of the L-tryptophan precursor, showing (i) the intramolecular migration of the carboxylate to C2 of the indole ring, (ii) transformation of the methylene carbon to a methyl group and (iii) elimination of the α-carbon and its associated amino group. The feeding of L-[$^{2}H_{8}$]-tryptophan also supported this conclusion by allowing the production of [$^{2}H_{6}$]-MIA (**5**) (HR-ESI-MS *m/z* calculated for $C_{10}D_{6}H_{2}O_{2}N$ = 180.09371, found 180.09343), and the feeding also excluded the participation of the benzene ring in this conversion (given no change in deuterium substitutions, **Scheme 1**). A 0.04-p.p.m. upfield shift (from δ 2.48 relative to CH_3) of the signal that corresponds to the CHD_2 protons was found in the ^{1}H NMR spectrum of **5** (**Supplementary Fig. 8**), ruling out adjacent hydrogen transfer, either from Cα or from C2 on the indole ring, to provide the third hydrogen atom of the methyl group. Further, using L-[$^{2}H_{8}$]-tryptophan as the substrate in an *in vitro* assay of NosL-catalyzed reactions showed that the coproduced AdoH (**2**) (HR-ESI-MS *m/z* calculated for $C_{10}H_{13}N_{5}O_{3}$ = 252.10967, found 252.10983) was not deuterium labeled. This clearly

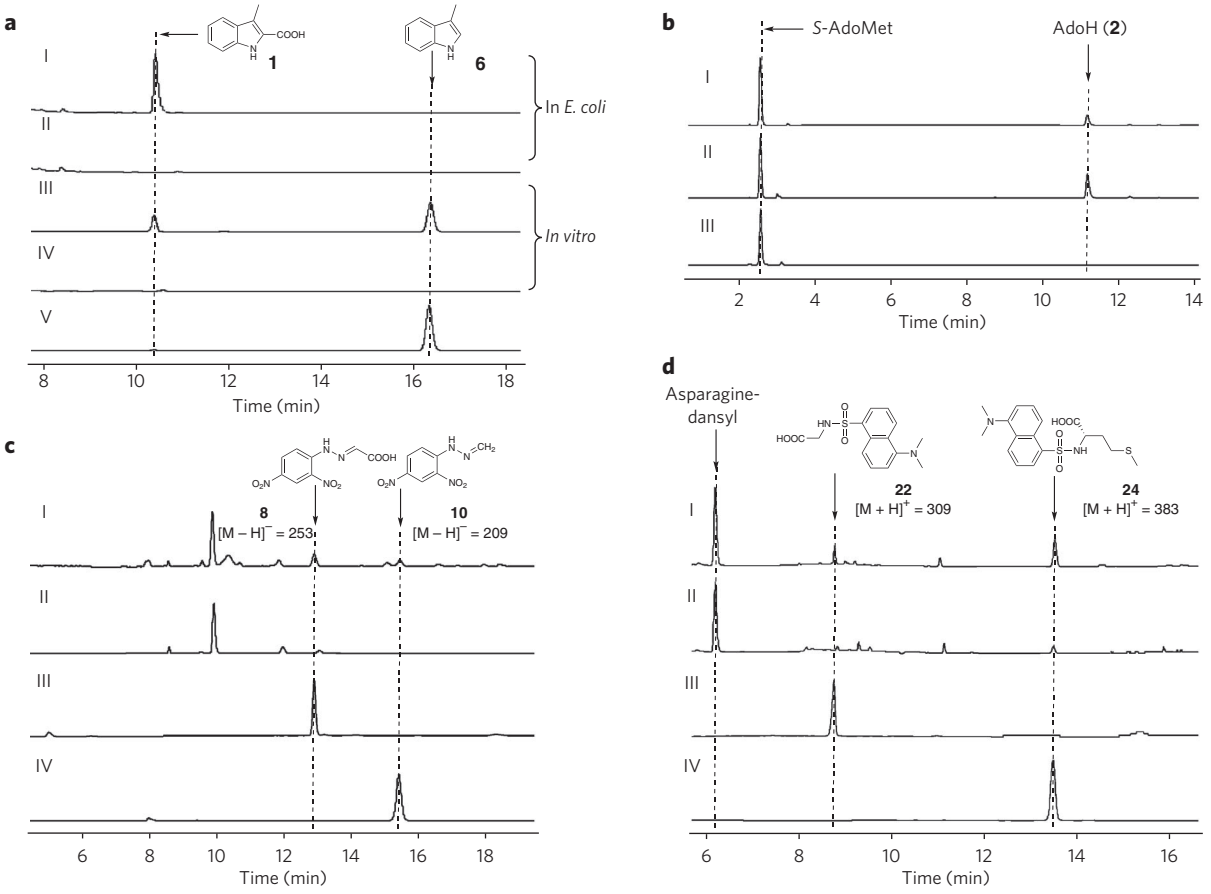


Figure 2 | Characterization of NosL-catalyzed reaction. All the examinations were performed at least in duplicate (each had at least two parallel samples). (**a**) Validation of NosL as a MIA synthase. *In vivo* **1** production in the *E. coli* strains, including SL4101 harboring *nosL* (**I**) and SL4100 carrying the vector alone (**II**), and *in vitro* conversion of L-tryptophan to **1** and the shunt product **6** in the presence (**III**) and in the absence (**IV**) of *S*-AdoMet, with the authentic **6** as a standard (**V**). (**b**) Consumption of *S*-AdoMet given the absence (**I**) and the presence (**II**) of L-tryptophan, in contrast to the results when NosL is omitted from the reaction mixture (**III**). (**c**) Identification of the products with DNP derivatization by adding (**I**) and by omitting (**II**) the substrate L-tryptophan, when authentic **7** (**III**) and **9** (**IV**) were subjected to DNP derivatization to generate the control derivatives. (**d**) Identification of the putative amino acid products with dansyl chloride derivatization by using asparagine as an internal standard for quantitative analysis. Production in the presence (**I**) and in the absence (**II**) of L-tryptophan while authentic **21** (**III**) and **23** (**IV**) were subjected to dansyl derivatization to generate the control derivatives for qualitative analysis.

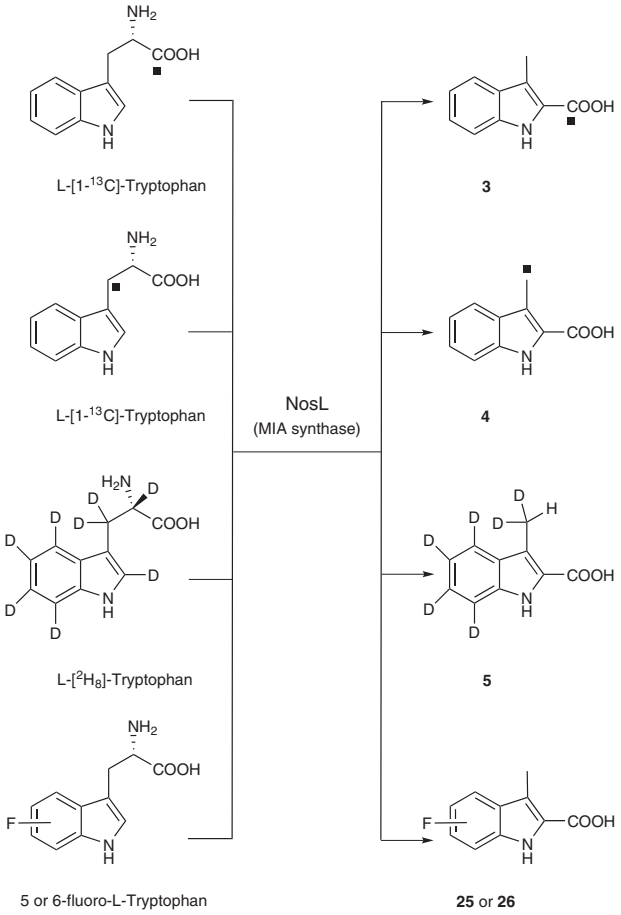
indicates that the Ado•-initiated rearrangement of the carbon chain does not start with a C-H bond cleavage on L-tryptophan to generate the substrate radical.

Determination of the coproduct and shunt products

To probe the individual steps in **1** formation, we carried out extensive survey of the product profile in NosL-catalyzed *in vitro* conversion. HPLC analysis of the reaction mixture revealed the first shunt product (**Fig. 2a**), which was deduced to be 3-methylindole (**6**) upon further GC-MS analysis with an authentic compound as the standard (**Supplementary Fig. 9**). Supplementation with 2,4-dinitrophenyl hydrazine (DNP) for derivatization led to identification of the second shunt product as glyoxylate (**7**) (corresponding to **8**, the derivative 2-(2,4-dinitrophenyl) hydrazonoacetate, **Fig. 2c** and **Supplementary Fig. 10**). Thus, the first Cα-Cβ cleavage of L-tryptophan appears to provide the 3-methylindole part, whereas the readdition-coupled, second cleavage may take place on the separated 2C-N unit to furnish the 2-carboxylate group. Consistent with this prediction, upon a similar derivatization, we detected the production of formaldehyde (**9**) (corresponding to **10**, the derivative 1-(2,4-dinitrophenyl)-2-methylene hydrazine, **Fig. 2c** and **Supplementary Fig. 11**), which most likely comes from the α-carbon of L-tryptophan.

Quantitative analysis of NosL-catalyzed *in vitro* reaction

We chose three representative products, including **1** and 3-methylindole (**6**) from L-tryptophan and AdoH (**2**) from *S*-AdoMet, to quantitatively evaluate NosL-catalyzed *in vitro* conversion (**Supplementary Fig. 4**). The yields of the products were enzyme-dose dependent, as the increase of the concentration of NosL accordingly improved the production of **1**, **6** and **2** (**Supplementary Fig. 4a**). Their productions at 25 °C were relatively constant in the presence of 1 mM dithionite, 500 μM L-tryptophan and 1 mM *S*-AdoMet, showing that about 39 ± 3 μM **1**, 140 ± 20 μM **6** and 381 ± 30 μM **2**, with a ratio of around 1:3.6:9.8, were produced by 20 μM NosL catalysis over a 2-hour period (**Supplementary Fig. 4b**). For conversion of the substrate L-tryptophan under these conditions, one molecule of NosL produced approximately two molecules of **1** along with seven molecules of **6**. The production of AdoH was consistently higher, showing a yield ratio around 2.1:1 for **2**, corresponding to a sum of **1** and **6**. For optimizing the reaction, we speculated that the degree of reduction in the reaction mixture may have an impact on the proportion of **1**. We therefore measured the effect of the concentration of the chemical reductant dithionite on the yields of **1** and **6** (**Supplementary Fig. 4c**). Intriguingly, **6** production was highly dependent on dithionite concentration, whereas the yields of **1** were relative constant, around



Scheme 1 | NosL-catalyzed carbon chain reconstitution of L-tryptophan shown by labeling patterns and generation of fluorinated MIA analogs. The solid rectangle shows the ^{13}C-labeled carbon atom. D, deuterium. F, fluorine.

30–42 μM in all assays. In particular, when 100 μM dithionite was used, the ratio of **1** to **6** can be improved to around 3:1.

Prediction of the Cα-Cβ bond cleavage manner

Inspired by identification of 3-methylindole (**6**) and glyoxylate (**7**) as the shunt products, we proposed that the Ado•-initiated carbon chain reconstitution of L-tryptophan may begin with a Cα-Cβ bond cleavage during NosL-catalyzed **1** formation. Depending on the bond-breaking pattern—that is, whether it is via homolysis or via heterolysis—there are two sets of hypothetical intermediates that share a common fate in generating the shunt product pair (**Fig. 3**). Thus, the density functional theory (DFT) calculation was performed to differentiate between these two possibilities (**Fig. 3** and **Supplementary Figs. 12** and **13**). Although the direct cleavage of the L-tryptophan neutral radical (**11**) as outlined in Path 1 leads to the generation of the intermediates **12** and **13** (heterolytic) or **14** and **15** (homolytic), Path 2 indicates that the cleavage accompanied by an intramolecular hydrogen migration results in the production of the intermediates **16** and **17** (heterolytic) or **18** and **19** (homolytic). Given that the intermediate **11** may get a proton from the environment, the model shown in Path 3 is also considered, indicating the generation of the intermediates **16** and **20** (heterolytic) or **18** and **15** (homolytic). At all events the homolytic products are lower in energy than their corresponding heterolytic products, suggesting that the homolysis was thermodynamically favored to form 3-methyleneindole and glycyl radical.

To experimentally support the hypothesis, we attempted to probe the putative intermediate in the NosL-catalyzed reaction by using a derivatization-associated, rapid-quench method previously used for analyzing the glutamate mutase–catalyzed reaction. Glutamate mutase, an Ado•-producing adenosylcobalamin (AdoCbl, coenzyme B_{12})-dependent protein, has been demonstrated to convert L-glutamic acid into glycyl radical (trapped as glycine by using this quench technique) and acrylate during L-*threo*-3-methylaspartate formation[18–20]. The NosL-catalyzed reaction was quenched at 10 sec before derivatization with dansyl chloride. HPLC-MS analysis showed the production of glycine (**21**) (corresponding to the derivative glycine-dansyl, **22**, **Fig. 2d** and **Supplementary Fig. 14**), as well as methionine (**23**) (corresponding to the derivative methionine-dansyl, **24**, **Fig. 2d** and **Supplementary Fig. 15**) deriving from *S*-AdoMet. Using asparagine as an internal standard, the glycine species was observed in a yield of 2 ± 0.5 μM, equal to around 2.5% of the enzyme active site. Under highly reductive conditions, this species is presumed to derive from the immediately uncoupled intermediate, glycyl radical, consistent with the predicted manner of homolytic cleavage of the Cα-Cβ bond of L-tryptophan during **1** formation.

Substrate flexibility of NosL

The characterization of the NosL chemistry then prompted us to feed available analogs of L-tryptophan, including D-tryptophan and derivatives with different substitutions on the indole ring, into SL4101 to determine the substrate tolerance of NosL. Individual additions of most of these substrates had no effect on **1** production and failed to afford the expected **1** analogs (**Supplementary Table 3**). These indicate the stringent substrate specificity of NosL in stereochemistry and in functionalization of the indole ring. In contrast, feeding of 5- or 6-fluoro-DL-tryptophan (only the L-configuration isomer can be used as the substrate) led to generation of the distinct product with a yield approximately 20% of the concomitant **1** production (**Supplementary Fig. 16**). Spectroscopic analyses, including HR-ESI-MS and ^{1}H, ^{13}C and ^{19}F NMR (**Supplementary Fig. 17**), clearly established these compounds as corresponding to 5-fluoro-MIA (**25**) and 6-fluoro-MIA (**26**), respectively (**Scheme 1**).

Generation of a fluorinated NOS analog

To determine whether or not the fluorinated MIA (**1**) can be incorporated as an intermediate *in vivo*, we fed 5-fluoro-DL-tryptophan into the NOS-producing strain *S. actuosus*. HPLC-MS analysis showed a dramatic decrease in NOS production (20–25% relative

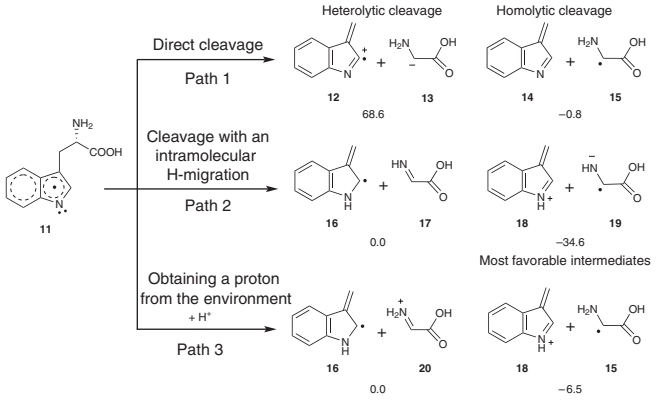


Figure 3 | Different patterns for cleaving the Cα-Cβ bond of L-tryptophan neutral radical 11. The DFT calculation–based relative free energies (including solvent effect ΔG_{sol}) are in kcal per mol. In Path 1 and Path 2, the energy of **16** + **17** is used as the reference zero energy, whereas in Path 3 the energy of **16** + **20** is used as the reference.

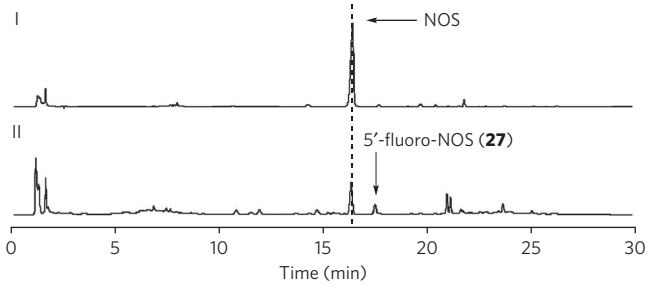


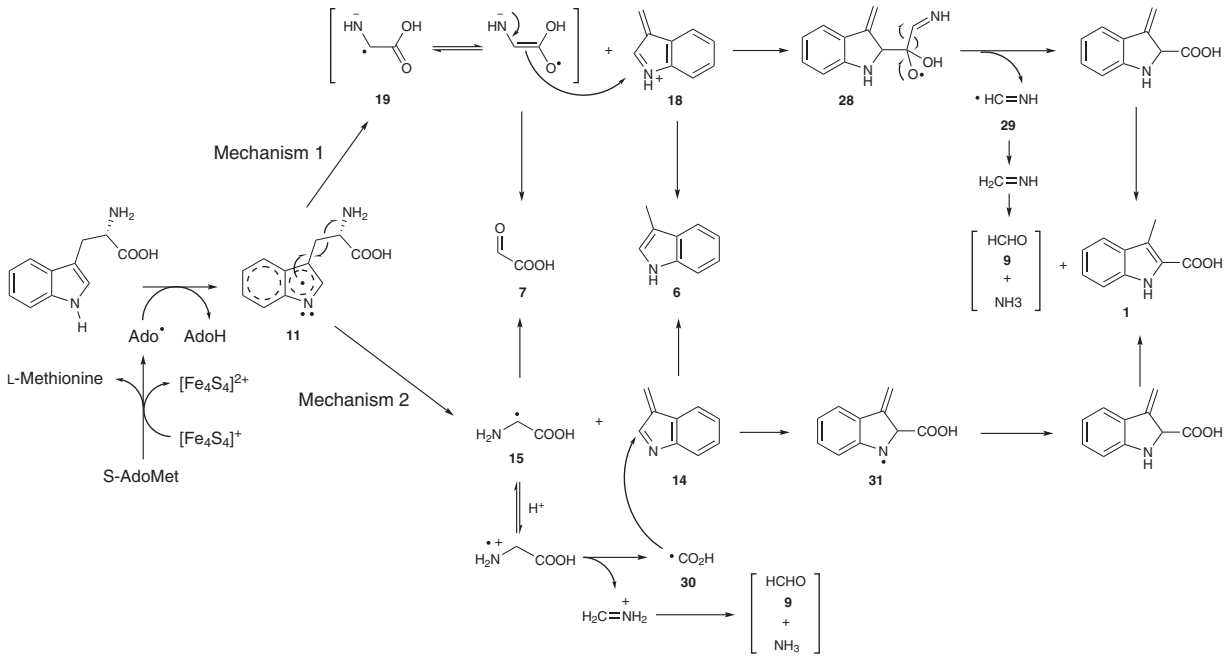
Figure 4 | Production of NOS and fluorinated thiopeptide. HPLC analysis of the fermentation cultures of *S. actuosus* without supplementation (**I**) and with supplementation with 5-fluoro-DL-tryptophan (**II**). All the examinations were performed in duplicate (each had two parallel samples).

to that of the wild-type strain, **Fig. 4**), presumably because of competitive inhibition of biosynthetic enzymes in the substrates. A new product, with UV spectra similar to NOS, was clearly observed, showing a yield approximately 20% of the concomitant NOS production. According to the molecular formula, $C_{51}H_{42}FN_{13}O_{12}$ (HR-ESI-MS *m/z* calculated = 1,238.13114, found 1,238.12959), this compound was deduced to be a NOS analog substituted by a fluorine atom at C5′ of the indole ring (**Fig. 1a** and **Supplementary Fig. 18**). For structural elucidation, it was purified and then subjected to comparative spectroscopic analysis with NOS, showing that the only difference was in the indole moiety. ^{19}F NMR and 1D and 2D NMR spectra further supported this structure assignment of 5′-fluoro-NOS (**27**, **Supplementary Fig. 19** and **Supplementary Table 4**). The effect of the 5′-fluoro-substitution on antibacterial activity was consequently evaluated by bioassays against the test strain *Bacillus subtilis*, showing the minimum inhibitory concentration at 0.004 μM ml^{-1} for **27** to that at 0.008 μM ml^{-1} for NOS (**Supplementary Table 5**). Notably, there has yet to be a halogenated member of the naturally occurring thiopeptide antibiotics, which number over 80 compounds reported to date[1].

DISCUSSION

The radical *S*-AdoMet superfamily currently comprises thousands of proteins that participate in numerous biochemical processes in animals, plants and microorganisms[17,21–24]. These proteins share a common mechanism to generate a powerful oxidant, Ado•, thereby initiating highly diverse transformations relevant to DNA repair and in the biosynthesis of vitamins, coenzymes and antibiotics. Biochemical mechanisms for most of such conversions have not been characterized, owing to a lack of information about the enzyme functions and radical-mediated transformations. Focusing on the characterization of NosL and NosL-catalyzed reactions, we herein unveiled a radical-mediated, unusual fragmentation-recombination process, which represents a key step in understanding radical *S*-AdoMet enzyme-catalyzed complex structure rearrangements.

We propose a mechanism for NosL-catalyzed reaction, as described below. Distinct from the widely found C-H bond cleavage in the reactions controlled by known radical *S*-AdoMet enzymes[17], Ado• in NosL catalysis may abstract the hydrogen atom from N_1 on the indole ring of L-tryptophan to generate **11**, the neutral substrate radical. The homolysis is thermodynamically favorable, leading to the Cα-Cβ bond cleavage for the first fragmentation, which gives the transient units 3-methyleneindole and glycine radical. Taking a simultaneous proton transfer into account, the resulting cationic 3-methyleneindole (**18**) and glycine radical (**19**) are more stable, facilitating their subsequent readdition to generate the radical intermediate **28** (**Scheme 2**, Mechanism 1). Elimination of the Cα-N unit (giving the methanimine radical **29**) from **28** can proceed to furnish the 2-carboxylate group to yield **1**, and the hydrogen rebound to **29** results in the production of CH_2=NH, which is hydrolyzed in the aqueous medium to form formaldehyde (**9**) and ammonia. Alternatively, according to a glycine decarboxylation–based free-radical mechanism proposed previously[25], the second fragmentation on the separated 2C-N unit (glycyl radical **15**) could precede the readdition, resulting in a direct reposition of the carboxylate at C2 of the indole ring (**Scheme 2**, Mechanism 2): (i) the protonated radical **15** may undergo a C-C bond cleavage to generate carboxyl radical **30**; and (ii) the addition of **30** to



Scheme 2 | Mechanistic hypothesis for NosL-catalyzed conversion. For Mechanism 1, the recombination may take place between cationic **18** and radical **19** followed by elimination of the Cα-N unit from the resulting radical **28**, and for Mechanism 2, the first separated radical **15** could undergo the second fragmentation to generate carboxyl radical **30** before the addition onto **14**.

3-methyleneindole **14** can yield radical **31**, which, when hydrogen is rebound, eventually leads to the production of **1**.

NosL-catalyzed *in vitro* conversion produced **1** along with two shunt products, 3-methylindole (**6**) and glyoxylate (**7**), which may originate from the intermediate pair 3-methyleneindole and glycyl radical, respectively. We proposed that the biotransformation after a Cα-Cβ bond cleavage, such as the inefficient recombination, may serve as a limitation step in NosL-catalyzed *in vitro* process. The overproduced intermediates could be released from the active site of NosL into the aqueous medium: while 3-methyleneindole is aromatized by reduction to **6**, glycyl radical is rapidly degraded to **7** and ammonia. Organic radicals with one unpaired electron have been known to be highly active and unstable. For instance, if the putative substrate-based glycyl radical (**Scheme 2**) fails to be further processed (to be added onto the intermediate 3-methyleneindole, as proposed), it may lose an electron to generate dehydroglycine and then undergo hydrolysis for glyoxylate production. A similar result has been found in the *in vitro* reaction catalyzed by ThiH[26]. Without the associated components for turning over glycyl radical or dehydroglycine in thiazole formation, ThiH catalyzes the Cα-Cβ bond cleavage of L-tyrosine and gives *p*-cresol and glyoxylate as the products.

Substrate fragmentation is known in Ado•-induced biotransformations, as exemplified by ThiH and by newly characterized HydG (24% identity to NosL)[27], which catalyzes the L-tyrosine cleavage to generate *p*-cresol and cyanide. NosL, ThiH and HydG may share a common paradigm to process the radical-based, aromatic amino acid substrates for the Cα-Cβ bond cleavage, despite the difference in the fate of the resulting glycyl radical (or dehydroglycine) intermediate. However, the fragmentation-recombination found in NosL chemistry is rare. To our knowledge, the only known example is glutamate mutase, the radical AdoCbl–dependent protein that converts L-glutamic acid to the β-methyl branched product (but without fragment elimination)[20,24]. This strategy might be used in certain Ado•-mediated structural rearrangements, such as the ThiC-catalyzed complex conversion of 5-aminoimidazole ribonucleotide to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate in thiamine biosynthesis[28].

In conclusion, we have uncovered NosL as a MIA synthase that catalyzes an unprecedented carbon side chain reconstitution of L-tryptophan. The radical-mediated, unusual fragmentation-recombination process may be general for radical *S*-AdoMet protein-catalyzed structural rearrangements in certain uncharacterized biotransformations. Taking advantage of the substrate tolerance of NosL, regiospecific fluorination of NOS could be achieved via a modified MIA intermediate. The NOS analog produced in this fashion and bearing fluorine at the 5′ position showed improved antibacterial activity. This application of combinatorial biosynthesis, enabled by our elucidation of NosL chemistry, complements recent advances in understanding sequence permutations of the precursor peptide[29,30].

METHODS

Bacterial strains, plasmids and primers. Please see **Supplementary Tables 1** and **2**.

Materials. Please see **Supplementary Methods**.

Production of MIA *in vivo*. Construction of the recombinant strain SL4101 for expressing *nosL* is described in **Supplementary Methods**. SL4101 was cultured in LB medium. Once the cell optical density (OD) reached 0.6–0.8 at 600 nm, the $Fe(NH_4)_2(SO_4)_2$ solution was added into the culture broth to a concentration of 50 μM, and then the cells were incubated on ice for 10 min. After addition of isopropyl-β-D-thiogalactopyranoside (100 μM in final), *nosL* expression had been induced at 25 °C for 6–8 h. After the cells were harvested by centrifugation for the following experiments (see below), the supernatant was directly subjected to HPLC analysis for product examination. SL4100 carrying the vector pET28a alone was used as the control in the parallel analytic process. Site-specific mutagenesis for exploring the cofactor-binding nature of NosL is described in **Supplementary Methods**.

Time course–based analysis of MIA production *in vivo*. The harvested SL4101 cells described above were resuspended in a nutrient-insufficient medium (yeast extract 0.1% (w/v), tryptone 0.5% (w/v), glucose 0.4% (w/v), NaCl 0.5% (w/v), glutamine 0.05% (w/v), K_2HPO_4 0.2% (w/v), $MgSO_4$ 0.01% (w/v) and $FeSO_4$ 0.001% (w/v)), with $OD_{600\,nm}$ of 0.6–0.8. Variable amino acids, including L-tryptophan, L-serine and L-glycine, were individually added to the broth (5 mM in final) before further incubation at 28 °C. MIA production was measured by HPLC at 0.5, 1, 2, 3, 4, 10 or 20 h. The MIA production is approximately linear with respect to time in initial 4 h, and the apparent rate was accordingly estimated.

Feeding of isotope-labeled L-tryptophans and variable L-tryptophan analogues. The procedure is similar to that of the time course–based analysis for MIA production, except the individual supplementation of isotope-labeled substrates (that is, L-[$^2H_8$]-tryptophan, L-[1-^{13}C]-tryptophan and L-[3-^{13}C]-tryptophan), D-tryptophan and derivatives with different substitution on the indole ring (that is, 1-methyl-L-tryptophan, 2-methyl-DL-tryptophan, 4-methyl-DL-tryptophan, 6-methyl-DL-tryptophan, 5-hydroxyl-L-tryptophan, 5-methoxy-DL-tryptophan, 5-bromo-DL-tryptophan, 5-fluoro-DL-tryptophan and 6-fluoro-DL-tryptophan) to a concentration of 1 mM before further incubation at 28 °C for 8 h. The production of labeled or functionally substituted MIAs was monitored by HPLC-MS.

Structural characterization of MIA, its analogs and a new NOS analog by feeding 5-fluoro-DL-tryptophan into the NOS-producing strain is summarized in **Supplementary Methods**.

Reconstitution of NosL. Production and anaerobic purification of NosL are summarized in **Supplementary Methods**. A previously reported procedure[31] was modified to reconstitute the active enzyme. Dithiothreitol was added to the purified protein (10 mM final). Under this reductive condition, $Fe(NH_4)_2(SO_4)_2$ solution (50 mM) was added carefully into the suspension to a final concentration of 500 μM. After 10 min, Na_2S solution (50 mM) was added in the same way to a concentration of 500 μM. The solution had been incubated on ice for 5–7 h. The resulting dark brown suspension was then subjected to desalting on a column (10-DG, Bio-Rad) that was pre-equilibrated with the elution buffer (50 mM Tris-HCl, 25 mM NaCl, 10 mM dithiothreitol and 10% (v/v) glycerol, pH 8.0). The colored fraction was collected and concentrated to 100 μM for *in vitro* assay. Protein characterization is described in **Supplementary Methods**.

Assay of the NosL activity *in vitro*. The 100 μl of reaction mixture contained 10 mM dithiothreitol, 500 μM dithionite, 200 μM *S*-AdoMet, 200 μM L-tryptophan (or L-[$^2H_8$]-tryptophan) and 20 μM reconstituted NosL in 50 mM Tris-HCl buffer (pH 8.0). Reactions were initiated by adding *S*-AdoMet to the 10-min preincubated mixture (as a negative control that contained the components except *S*-AdoMet); the mixture was then incubated at 25 °C for 2 h. To terminate the reaction, trifluoroacetic acid (TFA) was added to a final concentration of 10% (v/v) to inactivate the enzyme. After removal of the precipitates by centrifugation, the supernatant was subjected to HPLC-MS analysis. For evaluation of a natural reduction system, the chemical reductant dithionite was replaced by 0.5 mM NADPH, 50 μM flavodoxin and 20 μM flavodoxin reductase.

For GC-MS analysis, the supernatant was further extracted by ethyl acetate. For derivatization, DNP was added to the supernatant (10 mM final), which was further incubated at 37 °C for 30 min.

Quantitative analysis of product formation *in vitro*. The 100 μl of reaction mixture contained 10 mM dithiothreitol, 1 mM dithionite, 1 mM *S*-AdoMet and 500 μM L-tryptophan, in 50 mM Tris-HCl buffer (pH 8.0). The reconstituted NosL, varying in concentration (10 μM, 20 μM, 40 μM or 80 μM), was added into each reaction mixture. The 20 μM NosL catalysis was used for a time-course analysis (by terminating at 10 min, 30 min, 60 min, 90 min or 120 min). To measure the effect of dithionite concentration on the yields of MIA and 3-methylindole, each 100 μM of reaction mixture contained 20 μM reconstituted NosL, 10 mM dithiothreitol, 1 mM SAM, 500 μM L-tryptophan and dithionite varying in concentration (100 μM, 200 μM, 500 μM, 1 mM, 2 mM or 4 mM) in 50 mM Tris-HCl buffer (pH 8.0). The workups for the initiation, incubation and termination of reactions and for the analysis of products were identical to those described above.

Probing of the putative intermediate in NosL-catalyzed conversion. Trapping of glycine species in NosL-catalyzed conversion was performed according to a modified procedure[18]. The 100 μl of reaction mixture contained 20 mM dithiothreitol, 2 mM dithionite, 1 mM *S*-AdoMet, 1 mM L-tryptophan and 80 μM reduced NosL in 50 mM Tris-HCl buffer (pH 8.0). Reactions were initiated by adding *S*-AdoMet to the 10-min preincubated mixture (as a negative control containing the components except *S*-AdoMet); the mixture was mixed immediately and then terminated at 10 sec by addition of TFA to a final concentration of 15% (v/v) to precipitate the enzyme. After removal of the precipitate by centrifugation, Na_2CO_3 (1.5 M final) and dansyl chloride (1 mM final) were added to the supernatant, which was further incubated at 50 °C for 60 min. We then added 20 μl of TFA to the solution, and the dansyl derivatives were subjected to HPLC-MS analysis. For quantifying the yield of glycine species, asparagine (20 μM) was used as an internal standard.

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Author contributions

Q.Z., D.C., Y.Y. and L.D. carried out the experiments; Y.L. performed the theoretical calculations; Q.Z., B.S. and W.L. analyzed the data and wrote the paper; and W.L. designed the research. All authors discussed results and approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

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