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1,2,3-Triazine formation mechanism of a fairy chemical 2-azahypoxanthine in the fairy ring-forming fungus *Lepista sordida*†

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2-Azahypoxanthine (AHX) was first isolated from the culture broth of the fungus *Lepista sordida* as a fairy ring-inducing compound. It has since been found that a large number of plants and mushrooms produce AHX endogenously and that AHX has beneficial effects on plant growth. The AHX molecule has an unusual, nitrogen-rich 1,2,3-triazine moiety of unknown biosynthetic origin. Here, we establish the biosynthetic pathway for AHX formation in *L. sordida*. Our results reveal that the key nitrogen source that is responsible for the 1,2,3-triazine formation is reactive nitrogen species (RNS), which is derived from nitric oxide (NO) produced by NO synthase (NOS). Furthermore, RNS are also involved in the biochemical conversion of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5'-monophosphate (AICAR) to AHX-ribotide (AHXR), suggesting that a novel biosynthetic route that produces AHX exists in the fungus. These findings demonstrate a physiological role for NOS in AHX biosynthesis as well as in biosynthesis of other natural products containing a nitrogen-nitrogen bond.

Introduction

"Fairy rings" are a phenomenon in which ring-shaped overgrowth and/or growth-suppression of grass in floors of woodlands, agricultural or amenity grassland occurs. This is due to an interaction between the plant and a mushroom-forming fungus. The term, which derives from myths in the Middle Ages, may also refer to the fruiting bodies of the fungus that occasionally appear on the rings.[1] In 2010, we discovered two plant growth regulators from the culture broth of one of the fairy ring-forming fungi, *Lepista sordida*.[2, 3] One compound, 2-azahypoxanthine (AHX), is a growth promoter, while the other, imidazole-4-carboxamide (ICA), is a growth inhibitor. A metabolite of AHX that is commonly produced by plants, 2-aza-8-oxohypoxanthine (AOH), was later found from AHX-treated rice. Like AHX, AOH showed plant growth-regulating activity.[4] These three compounds are collectively termed "fairy chemicals" (FCs).[5] FCs exhibit growth-regulating activity not only against turfgrass but also against all kinds of plants tested, regardless of families they belong to.[2-4] Furthermore, grain yields of rice, wheat, and other crops are significantly increased by treatment with each of the three FCs in greenhouse and/or field experiments. FC-treated plants acquire greater tolerance to abiotic stress, suggesting FCs may find practical applications in agriculture.[2-4, 6]
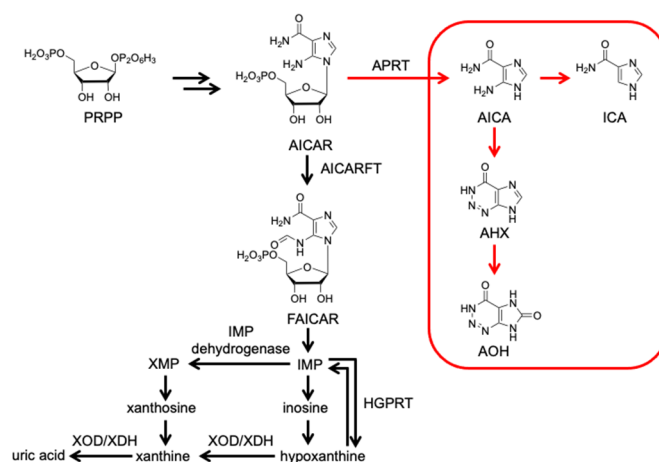

Fig. 1 Purine metabolic pathway common in plants, animals, and microorganisms, including FCs producing route. The route in black is cited from KEGG (Kyoto Encyclopedia of Genes and Genomes). The biosynthetic pathway of FCs is shown in red. Two consecutive arrows in

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the same direction represent multistage enzyme reactions. PRPP, phosphoribosyl pyrophosphate; AICARFT, AICAR formyltransferase; FAICAR, *N*-formyl-AICAR; IMP, inosine monophosphate; XMP, xanthosine monophosphate; XOD, xanthine oxidase; XDH, xanthine dehydrogenase.

FCs are produced endogenously in various plants as well as in mushrooms.[4, 7-10] The biosynthetic pathway of FCs in plants and mushrooms has been partially elucidated, revealing that they are products of the purine metabolic pathway (Fig. 1).[4, 7-11] When *L. sordida* cultures are fed 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5'-monophosphate (AICAR), which is a precursor of fundamental metabolites such as xanthine, hypoxanthine and uric acid in the purine metabolic pathway, AICAR is consumed and AHX accumulates.[11] AICAR is converted by recombinant adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) from *L. sordida* to 5-aminoimidazole-4-carboxamide (AICA).[10] AICA is a biosynthetic precursor of AHX and ICA in rice and *L. sordida*. This was shown by the detection of double-^{13}C-labeled AHX and ICA in the extracts of both species after incubation with double-^{13}C-labeled AICA.[9, 10, 12] However, the enzyme(s) responsible for the conversion of AICA to AHX and ICA has not been identified.

Nitric oxide (NO) is a ubiquitous intracellular and intercellular signaling messenger that regulates many physiological processes in various living organisms. For example, NO confers tolerance to oxidative stress by enhancing intracellular anti-oxidative activity in mammals and plants.[13, 14] In animals and microorganisms, NO is mainly produced by NO synthase (NOS; EC 1.14.13.39), which catalyzes the reaction from L-Arg to L-citrulline and NO using NADPH as an electron donor and O_2 as a co-substrate.[15-18] Three NOS isozymes have been identified in animals: constitutive neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS).[19]

During chemical synthesis of AHX, AICA is a precursor. AICA reacts with $NaNO_2$ under an acidic condition to form an unstable intermediate, 4-diazo-4*H*-imidazole-5-carboxamide (DICA), that is converted to AHX after treatment with NH_3 or MeOH (Fig. S1A†).[20-23] The biosynthetic pathway from AICA to AHX in *L. sordida* is likely to be similar, but the nitrogen source for the reaction is unknown. The goal of this study is to identify the nitrogen source and the mechanism for forming the unique 1,2,3-triazine moiety during AHX biosynthesis in *L. sordida*. In a recent analysis of the *L. sordida* genome, we discovered seven *NOS* genes.[24] We hypothesized that LsNOS enzymes provide NO or nitrite used in the conversion of AICA to DICA, and that DICA then undergoes intramolecular cyclization to form the unique 1,2,3-triazine moiety in AHX. Here we report that LsNOS enzymes mediate the 1,2,3-triazine formation via NO-derived metabolites as reactive nitrogen species (RNS).

Results and discussion

L-Arg promotes FCs accumulation in *L. sordida*
FCs have a purine-like skeleton. Purine skeletons such as xanthine and hypoxanthine are constructed with L-Asp, L-Gln, and Gly in living organisms without exception (Fig. S2†). In a

previous feeding study, [1,2-[13]C$_2$] Gly was added to *L. sordida* cultures, and both of the ^{13}C of the amino acid were incorporated into the purine-like structures of AHX and ICA. The result suggested that both compounds are biosynthesized from the purine metabolic pathway in this fungus.[10]

We hypothesized that the key nitrogen source from AICA to AHX might also come from an amino acid(s). Therefore, we performed a feeding study with 20 kinds of proteinogenic amino acids. *L. sordida* was incubated with each amino acid for 5 weeks and the culture broth was analyzed by reverse-phase HPLC (RP-HPLC). The amounts of AHX and ICA in control (non-fed treatment) were 187 ± 22 and 17 ± 6 μM, respectively (Fig. 2). AHX abundance after treatment with L-Arg was more than three-fold higher (598 ± 119 μM; Fig. 2A). ICA abundance after treatment with L-Arg (29 ± 7 μM) also tended to be higher than the control (~1.7-fold), although this was not a statistically significant difference (Fig. 2B). None of the other 19 amino acids tested caused a statistically significant change in AHX or ICA production.
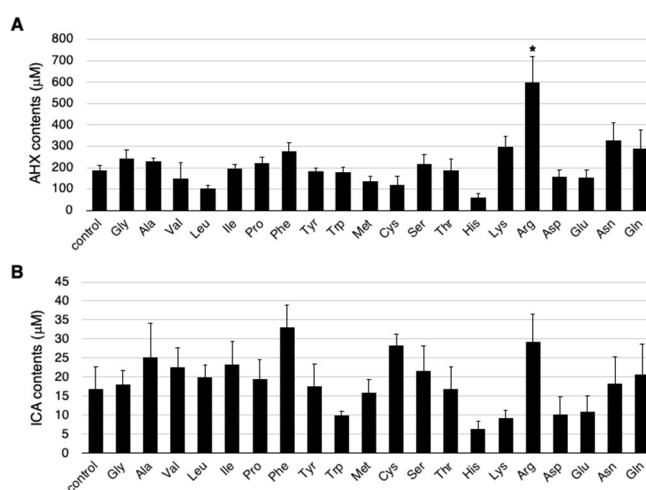

Fig. 2 Effect of 20 kinds of amino acids on AHX and ICA production in *L. sordida*. All these amino acids are L-isomers except for glycine. (A) AHX concentration in culture broth. (B) ICA concentration in culture broth. *L. sordida* mycelia were cultured for 5 weeks and the culture broth was assayed by RP-HPLC. Mean ± standard error (n = 3). *, *p* < 0.05 t-test comparison to control.

Nitric oxide promotes AHX synthesis in *L. sordida* mycelia and *in vitro*
Based on this result, we hypothesized that NOS in *L. sordida* may generate a nitrogen donor from L-Arg that converts AICA to DICA (Fig. S1B). To investigate this, we cultured the mycelia with a NOS competitive inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), and/or L-Arg, and then quantified the amount of AHX in the culture broth by RP-HPLC. Over a 13-day time course, we observed that AHX levels were significantly reduced after five days of treatment with L-NAME compared to an untreated control sample (Fig. 3). L-Arg caused a significant increase in AHX abundance compared to a negative control after five days of treatment, but this increase was strongly suppressed by L-NAME (Fig. 3).
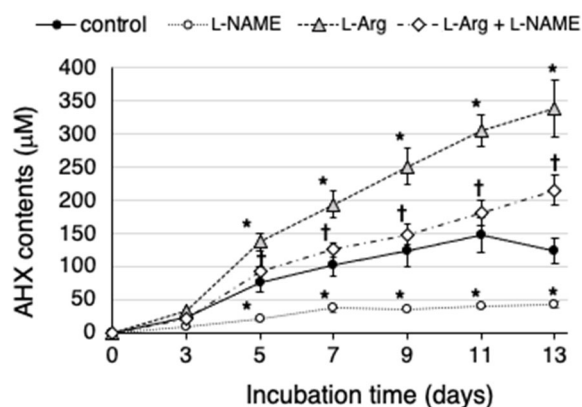
Fig. 3 Effect of L-NAME and L-Arg treatments on AHX production in *L. sordida*. The concentration of AHX in the culture broth in non-fed control (black circle), L-NAME only treatment (white circle), L-Arg only treatment (gray triangle), and L-Arg and L-NAME treatment (white rhombus) was quantified by RP-HPLC. Mean ± standard error (n = 3). Asterisks and daggers represent statistical significance of difference ($p < 0.05$, t-test) from the control and L-Arg treatment, respectively.

To date, approximately 200 kinds of N-N bond-containing natural products (including AHX and AOH) with various biological activities have been found. The involvement of NO in N-N bond formation has been widely proposed. However, there has been little direct evidence that NO generated by NOSs contributes to N-N bond formation.[25, 26] A previous labeling study utilizing double-^{13}C-labeled AICA recently demonstrated that AHX is biosynthesized from AICA in *L. sordida*.[10] IN this study, AICA was incubated with an NO donor, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5) in order to know whether AICA reacts with NO and is converted into AHX. As a result, AHX production by the reaction was clearly detected by LC-MS/MS (Fig. S3A†). On the other hand, when NOC5 was preincubated with an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), before adding AICA, the molecular ion peak of AHX was not detected by the analysis (Fig. S3B†).

The guanidino-nitrogen of L-Arg is incorporated into AHX via NO

In the conversion of L-Arg to L-citrulline and NO by NOS enzymes, the nitrogen atom of NO derives from a guanidino-nitrogen in the amino acid (Fig. S1B†).[24, 27] To test whether NO derived from L-Arg is incorporated directly into AHX, we incubated *L. sordida* with [guanidino-[15]N$_2$] L-Arg or unlabeled L-Arg. The culture broth was fractionated and analyzed for FCs by LC-MS/MS (negative mode) according to the previously developed method.[9] The fraction incubated with labeled L-Arg gave a heavy parent ion of AHX (*m/z* 137) along with the lighter native one (*m/z* 136) (Fig. S4A†). When incubated with unlabeled L-Arg, however, only the native parent ion peak was detected (Fig. S4B†). Then, AHX was purified from the fraction by repeated column chromatography and analyzed by LC-MS/MS. The AHX purified from the cultures incubated with the labeled L-Arg gave both the native and ^{15}N-labeled parent ions (Fig. S5A,B†). Unlabeled AHX (authentic standard) gave a daughter ion at *m/z* 80 derived from an imidazole ring (Fig. S5C†), and the purified

[15]N-labeled AHX also gave the same daughter ion (Fig. S5D†). These results and those described above (Fig. S3A†) suggest that N-2 in AHX is derived from the guanidino-nitrogen of L-Arg.

At least eight *NOS* genes are expressed in *L. sordida*

We next set out to determine which nitric oxide synthase(s) in *L. sordida* are most likely to participate in AHX biosynthesis. Previously, seven *NOS* genes were found in a draft genome assembly of the fungus that was estimated to cover ~75% of the genome.[24] We used RNA-seq data from *L. sordida* mycelium to improve the accuracy of gene predictions in the *L. sordida* draft genome. This led to the identification of eight *NOS* genes (*LsNOS 1–8* genes) and their protein-coding sequences (Genbank accession numbers GHP15256.1–GHP15263.1). Seven of the eight genes were present on scaffold18 (BIMQ01000018.1) within a range of 213 kb, of which two (*LsNOS1* and *LsNOS2*) and four (*LsNOS4* to *LsNOS7*) genes were tandemly arrayed. *LsNOS8* gene was present on scaffold60 (BIMQ01000060.1). Because the draft genome currently consists of 703 scaffolds, it is not known whether scaffold18 and scaffold60 are genetically linked.

To compare the expression of the eight *LsNOS* genes, we mapped RNA-seq reads to the *L. sordida* genome and calculated transcripts per million (TPM) values.[28] All *LsNOS* genes were moderately expressed (TPM, 2.2–373.5) compared with the median expression level (TPM, 22.7) and *LsActin* gene expression level (TPM, 1546.8) (Fig. S6†). The highest expressed gene was *LsNOS2* (TPM, 373.5), followed by *LsNOS8* (TPM, 133.8) and *LsNOS1* (TPM, 37.5).
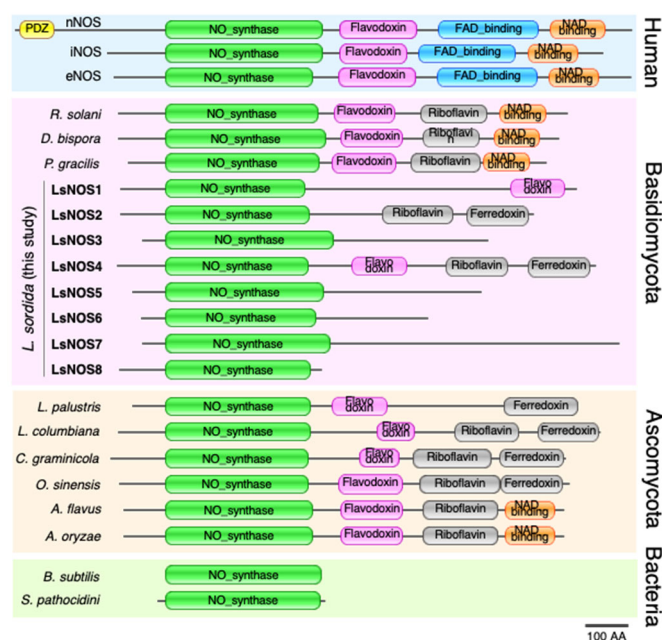

Fig. 4 Domain organization of NOS proteins of human, Basidiomycota, Ascomycota, and bacteria. The total and domain lengths of NOSs are indicated on the common scale as shown by the scale bar (100 amino acids). Pfam domains are shown in boxes of the following colors: yellow, PDZ (PF00595); green, NO_synthase (PF02898); pink, Flavodoxin_1 (PF00258); blue, FAD_binding_1 (PF00667); orange, NAD_binding_1 (PF00175). "Riboflavin synthase domain-like" (SSF63380) and "Ferredoxin reductase-like, C-terminal NADP-linked domain" (SSF52343) similar to FAD_binding_1 and NAD_binding_1, respectively, are indicated in gray boxes. Species and accession numbers of NOSs

used in this analysis are: *Homo sapiens* (nNOS, NP_000611.1; iNOS, NP_000616.3; eNOS, NP_000594.2), *Rhizoctonia solani* (EUC55953.1), *Dendrothele bispora* (THU86598.1), *Pterula gracilis* (TFK98355.1), *Lepista sordida* (LsNOS1–8, GHP15256.1–GHP15263.1), *Lepidopterella palustris* (OCK79887.1), *Letharia columbiana* (KAF6239642.1), *Colletotrichum graminicola* (EFQ35699.1), *Ophiocordyceps sinensis* (EQL02386.1), *Aspergillus flavus* (XP_002381643.1), *Aspergillus oryzae* (XP_001825673.2), *Bacillus subtilis* (O34453.2), *Streptomyces pathocidini* (QIQ51167.1).

NOS proteins in *L. sordida* have diverse and unusual *C*-terminal domain content

Most metazoan NOS proteins have a conserved domain organization consisting of the Pfam domains, in order, "Nitric oxide synthase, oxygenase domain" (NO_synthase, PF02898), "Flavodoxin_1" (PF00258), "FAD_binding_1" (PF00667), and "NAD_binding_1" (PF00175).[29-31] We used InterProScan (http://www.ebi.ac.uk/interpro/) to investigate which domains are present in the NOS proteins from *L. sordida* and other representative fungi from the Ascomycota and Basidiomycota (Fig. 4).[32]

Similar to metazoans, all fungal NOS proteins had an *N*-terminal NO_synthase domain. Although the Flavodoxin_1 domain was present in NOS proteins of the other Basidiomycota and Ascomycota, it was present only in LsNOS1 and LsNOS4 in *L. sordida*. Furthermore, no FAD_binding_1 domain was detected in NOS proteins of either Ascomycota or Basidiomycota. Instead, a "Riboflavin synthase domain-like superfamily" domain (SSF63380), which overlaps with the FAD_binding_1 domain in the SUPERFAMILY database, was detected in various fungal NOS proteins at the position corresponding to the FAD_binding_1 domain. This domain might have a similar function in fungal NOS proteins as the FAD_binding_1 domain, despite poor sequence conservation.[33] The NAD_binding_1 domain was also absent from the NOS proteins in *L. sordida* and several Ascomycota species. Instead, "Ferredoxin reductase-like, C-terminal NADP-linked domain" (SSF52343) was detected at the position corresponding to the NAD_binding_1 domain. Only LsNOS4 contained all four functional domains, indicating that NOS proteins in *L. sordida* have a poorly conserved domain organization compared to NOS proteins of other Basidiomycota and Ascomycota. In addition to having varied domain content, LsNOS proteins have diverse lengths. For example, LsNOS8 lacks a *C*-terminal region after the NO_synthase domain; this structure was only observed in bacterial NOSs. Also highly unusual, LsNOS1 has a *C*-terminal Flavodoxin_1 domain. While it is possible that some *NOS* genes in *L. sordida* are pseudogenes, we did not observe a correlation between the domain content and organization of LsNOS proteins and their gene expression level. Neither did we observe consistent *C*-terminal domain content among tandemly arrayed *LsNOS* genes.

The NOS oxygenase domain in LsNOS proteins is most similar to Ascomycota NOS

We investigated the evolution of the relatively large and unusual *NOS* gene family in *L. sordida*. We performed a phylogenetic analysis using the NOS_oxygenase domain

(Conserved Domain Database cd00575, https://www.ncbi.nlm.nih.gov/Structure/cdd/cd00575) of 57 NOS proteins from *L. sordida* and 37 other species (Table S1†). The phylogenetic tree based on this domain was divided into two major clusters: a general-type (including metazoan, bacteria, and filamentous fungi (Basidiomycota) other than *L. sordida*) and a fungal-type (including Ascomycota and *L. sordida*) (Fig. 5). All LsNOS proteins formed a monophyletic clade in the fungal-type cluster, suggesting that the family arose from gene duplication in the *L. sordida* lineage. The phylogenetic tree also showed that LsNOS4 is an early-branched, potentially ancestral NOS in *L. sordida*, which is consistent with its *C*-terminal domain organization being most similar to other fungal NOS proteins (Fig. 4). Interestingly, the expression level of *LsNOS4* was the lowest among the eight *LsNOS* genes (Fig. S6†). Furthermore, the most highly expressed genes, *LsNOS1*, *LsNOS2*, and *LsNOS8*, formed a monophyletic group branched from other LsNOS proteins (Fig. 5). LsNOS proteins formed a monophyletic group, suggesting that an unusual expansion and diversification of this family has occurred in the *L. sordida* lineage. The most similar NOS_oxygenase domains to those in LsNOS proteins were found in a wolf lichen, *Letharia columbiana* (Lecanoromycetes), and a freshwater ascomycete, *Lepidopterella palustris* (Dothideomycetes), which are phylogenetically distant species belonging to different classes. Their NOS proteins were also similar to LsNOS proteins in that they tended to have poor domain conservation (Fig. 4).

Fig. 5 Maximum likelihood phylogeny of the NOS_oxygenase domain of NOS proteins in Basidiomycota, Ascomycota, other eukaryotes, and bacteria. Amino acid sequences of the NOS_oxygenase domain were aligned using MAFFT and the phylogenetic tree was reconstructed using IQ-TREE with 1,000 replicates of the ultrafast bootstrap. The ultrafast bootstrap values higher than 75 are shown at the nodes. Scale bar

indicates the number of amino acid substitutions per site. 57 amino acid sequences of the NOS_oxygenase domain of species used in this analysis are listed in Table S1.

Several amino acids involved in L-Arg/NOHA binding are conserved in LsNOS proteins

Generally, NOS proteins catalyze two-step oxidation reactions of L-Arg to generate L-citrulline and NO, with formation of *N*-hydroxy-L-Arg (NOHA) as an catalytic intermediate.[15-18] To estimate L-Arg- and NOHA-binding abilities of LsNOS proteins, we analyzed the conservation of amino acids involved in L-Arg/NOHA-binding (Fig. S7†); a multiple sequence alignment of the NOS_oxygenase domain of eight LsNOS proteins, mouse iNOS (accession number, NP_035057.1), and *Bacillus subtilis* NOS (accession number, O34453.2) was performed. In mouse iNOS, Glu-371 is essential for binding the two guanidino nitrogens and α-amino group in L-Arg/NOHA, Trp-366 forms a hydrogen bond with the guanidino nitrogen, and Gln-257 interacts with the carboxy group in L-Arg/NOHA.[34-37] All three residues were conserved in LsNOS proteins. By contrast, Tyr-367, which in iNOS forms a hydrogen bond with the carboxy group in L-Arg/NOHA, was substituted to Phe in all LsNOSs.[34-37] This substitution was mainly observed in fungal NOSs of most Ascomycota except for the genus *Aspergillus*. Interestingly, although NOS proteins of Basidiomycota *Rhizoctonia solani* and *Pterula gracilis* are phylogenetically distant to Ascomycota NOS and LsNOS proteins, they have the same substitution. Other than fungi, the same substitution was found only in NOS of a slime mold, *Physarum polycephalum*.[38] Asp-376, which in iNOS forms a hydrogen bond with the L-Arg/NOHA carboxy group, was substituted to Asn in LsNOS1, LsNOS2, LsNOS4, LsNOS5, and LsNOS8.[34-37] This substitution was also observed in 48 amino acid sequences of the NOS_oxygenase domain (cd00575, https://www.ncbi.nlm.nih.gov/Structure/cdd/cd00575). The only distinct difference in the binding modes of L-Arg and NOHA is interaction made by the NOHA hydroxy group.[35, 37] Gly-365, which in iNOS forms a hydrogen bond between its peptide nitrogen and the NOHA hydroxy oxygen, was conserved in all LsNOS proteins.[35, 37]

LsNOS2 forms a 1,2,3-triazine moiety *in vitro* and formation of the ring occurs at low pH

We examined the enzymatic activity of the two most highly expressed *NOS* genes in *L. sordida*, *LsNOS2* and *LsNOS8*. Recombinant His-tagged enzymes (rLsNOS2 and rLsNOS8) were expressed in *E. coli*.

The rLsNOS8 enzyme was purified by Ni-affinity column chromatography, producing a single band in SDS-PAGE that agreed with its predicted molecular mass (54.5 kDa) (Fig. S8A†). Subsequently, the catalytic activity producing NO from NOHA of the enzyme was examined in the presence of hydrogen peroxide (H_2O_2).[26] This assay has been widely used for *in vitro* characterization of NOS proteins.[26] Incubation of the reaction mixture for 15 min at room temperature resulted in detection of about 48-fold more nitrite than is generated from NOHA non-enzymatically and spontaneously (Fig. S8B†).

Another recombinant enzyme, rLsNOS2, was purified by the same method, however, its activity was very low. Therefore, the

recombinant protein was refolded in the presence of hemin to yield the active enzyme. (Fig. S9A†).[39-41] The activity of the enzyme on NOHA was increased more than 23-fold by refolding, and it was about 3.6-fold higher than that of rLsNOS8 (Fig. S9B,C†).

However, neither rLsNOS2 nor rLsNOS8 showed activity against L-Arg (Fig. S10†). NO generation by mammalian NOSs involves electron transfer between reductase and oxygenase domains in a NOS molecule. Because rLsNOS2 and rLsNOS8 lack the reductase domain (*C*-terminal protein) in the molecule, their heme can only receive electrons from another donor protein (Fig. 4). Therefore, the rLsNOS oxidized NOHA to produce NO *in vitro* in the presence of an electron donor such as H_2O_2, similar to the bacterial NOS.[17]
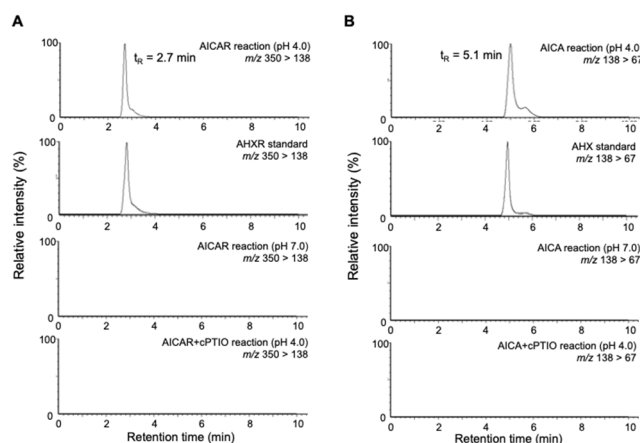

Fig. 6 *In vitro* enzymatic assembly of AHXR and AHX by rLsNOS2. (A) LC-MS/MS chromatograms of the reaction from AICAR to AHXR. (B) LC-MS/MS chromatograms of the reaction from AICA to AHX. The reaction mixture containing cPTIO (an NO scavenger) was used as a negative control. The reaction was done for 3 h under pH 4.0. The two compounds were detected in the positive mode. LC-MS/MS chromatograms indicate selected reaction monitoring for AHXR and AHX, respectively. t_R, retention time.

In order to examine whether rLsNOS2 can produce a 1,2,3-triazine moiety, the enzymatic reaction was analyzed by LC-MS/MS. In the presence of NOHA and H_2O_2, rLsNOS2 converted AICAR to AHX-ribotide (AHXR) and AICA to AHX. This activity was inhibited in the presence of the NO scavenger, cPTIO (Fig. 6). However, formation of the 1,2,3-triazine products was pH-dependent; the reactions occurred successfully at pH 4.0, but not at pH 7.0. This indicated that an unknown active molecule(s) other than NO must be involved in N-N bond formation during AHX biosynthesis. We propose that nitrosonium ion (NO^+) and dinitrogen trioxide (N_2O_3) are directly responsible for 1,2,3-triazine formation in AHX (Fig. 7); AHX was produced from AICA by rLsNOS2 when the reaction mixture was adjusted to pH 4.0 (Fig. 6B). Under acidic conditions, NO produced by rLsNOS2 can be easily converted into RNS such as N_2O_3 and/or NO^+. In the case of chemical NO donors such as NOC5, N_2O_3 is produced by a rapid reaction between NO and O_2.[26, 42] Therefore, N_2O_3 and NO^+ might be easily generated under a mild condition when an NO donor such as NOC5 is used, enabling AHX production from

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AICA. In addition, we found additional evidence for the effect of pH on AHX formation. When chemical reactions of AICA with $NaNO_2$ were examined at pH 5.0, 6.0, and 7.0, AHX was only produced at pH 6.0 and below (Fig. S11†). It has been known that NO_2^- can also change to N_2O_3/NO^+ under acidic conditions non-enzymatically and spontaneously (Fig. 7).[39] We also noted that the pH of *L. sordida* cultures gradually decreased to 6.0 or below over a three-week period (Fig. S12†). This pH change may affect AHX formation in the culture from AICA and nitrite.
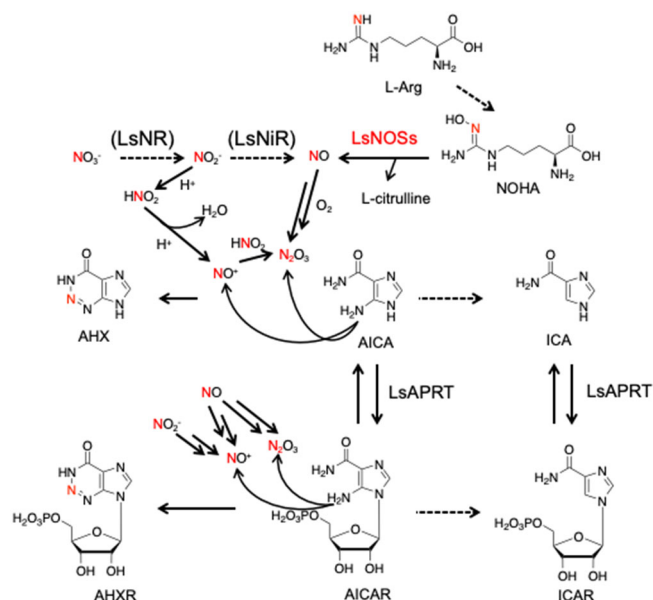

Fig. 7 Proposed biosynthetic pathways to AHX and ICA in *L. sordida*. The enzyme LsAPRT is described in the reference 11. The red letters in the routes are revealed in this study. Two consecutive arrows in the same direction represent multistage enzyme reactions. Dashed arrows represent unidentified biosynthetic pathways.

In addition, *L. sordida* produced ^{15}N-labeled AHX when ^{15}N-labeled $NaNO_2$ or ^{15}N-labeled $NaNO_3$ was added into the cultures (Fig. S13B†), suggesting that there are multiple nitrite supply routes to produce AHX. It is possible that nitrate reductase, which reduces nitrate to nitrite, contributes to AHX biosynthesis. In the draft genome sequence of *L. sordida,* two genes and one gene encoding nitrate reductase and nitrite reductase, respectively, have been found.[24, 43] However, the production of AHX after L-Arg treatment was double that of the sodium nitrite or nitrate treatments (Fig. S13A†).

Conclusions

We have revealed the unique formation mechanism of the 1,2,3-triazine in AHX in *L. sordida* (Fig. 7): NO-derived RNS, N_2O_3 and NO^+, could be the direct nitrogen source for the reaction with AICA. Our results in this study have expanded the physiological role of NOS proteins to include forming nitrogen donors that create N-N-bond-containing natural products. In addition, NO plays various important roles in living organisms. Therefore, this study will cause a stir in argument about the real active species of NO in living organisms.

Author Contributions

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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