JOURNAL OF NATURAL PRODUCTS

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Article

Biosynthesis of the Fairy Chemicals, 2-Azahypoxanthine and Imidazole-4-carboxamide, in the Fairy Ring-Forming Fungus *Lepista sordida*

Akinobu Ito, Jae-Hoon Choi, Hirohide Takemura, Mihaya Kotajima, Jing Wu, Shinji Tokuyama, Hirofumi Hirai, Tomohiro Asakawa, Hitoshi Ouchi, Makoto Inai, Toshiyuki Kan, and Hirokazu Kawagishi*

Cite This: https://dx.doi.org/10.1021/acs.jnatprod.0c00394

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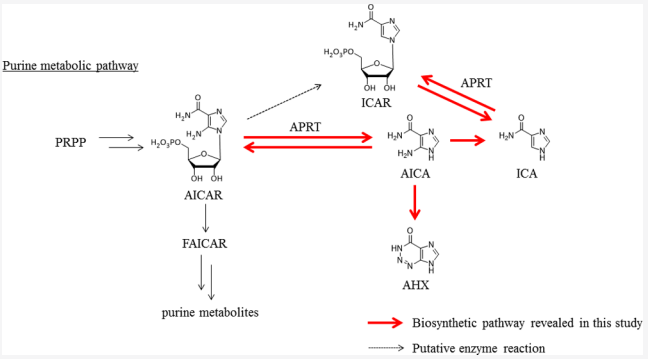
ABSTRACT: Fairy rings resulting from a fungus−plant interaction appear worldwide. 2-Azahypoxanthine (AHX) and imidazole-4-carboxamide (ICA) were first isolated from the culture broth of one of the fairy ring-forming fungi, *Lepista sordida*. Afterward, a common metabolite of AHX in plants, 2-aza-8-oxohypoxanthine (AOH), was found in AHX-treated rice. The biosynthetic pathway of the three compounds that are named as fairy chemicals (FCs) in plants has been partially elucidated; however, that in mushrooms remains unknown. In this study, it was revealed that the carbon skeletons of AHX and ICA were constructed from Gly in *L. sordida* mycelia and the fungus metabolized 5-aminoimidazole-4-carboxamide (AICA) to both of the compounds. These results indicated that FCs were biosynthesized by a diversion of the purine metabolic pathway in *L. sordida* mycelia, similar to that in plants. Furthermore, we showed that recombinant adenine phosphoribosyltransferase (APRT) catalyzed reversible interconversion not only between 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR) and AICA but also between ICA-ribotide (ICAR) and ICA. Furthermore, the presence of ICAR in *L. sordida* mycelia was proven for the first time by LC-MS/MS detection, and this study provided the first report that there was a novel metabolic pathway of ICA in which its ribotide was an intermediate in the fungus.



The rings, ribbons, or arcs of fungus-stimulated and/or fungus-suppressed plant growth, which often occur on floors of woodlands and agricultural grasslands, are generally called "fairy rings".[1] The term "fairy rings" derives from the myths and superstitions associated with this phenomenon in the Middle Ages. The fruiting bodies of fungi sometimes appear on the fairy rings, and it has been reported that about 60 kinds of mushroom-forming fungi form fairy rings all over the world.[2] Since the first scientific paper on fairy rings was published in 1675 and the subsequent research about them was reviewed in *Nature* in 1884, the real identity of the "fairy" had been unknown before our research.[3] The accepted notion of growth stimulation for fairy rings is that the fungal mycelia decompose the protein in the soil and the plant growth is promoted by absorption of accumulated nitrite and nitrate in the soil.[1−7] However, we questioned this notion and proposed the possibility that fairy ring-forming fungi produced specific plant growth regulators, and we tried to isolate the causative chemicals from one of the fairy ring-forming fungi, *Lepista sordida*.

L. sordida is widespread in northern temperate zones throughout the world and is one of the famous fairy ring-forming fungi in Japan; thus this fungus was chosen as a material for our chemical research.[8] In 2010, two plant growth-regulating substances, 2-azahypoxanthine (AHX) and imidazole-4-carboxamide (ICA), were isolated from the culture broth of *L. sordida* mycelia, and this was the first reported isolation of both of the compounds from a natural source.[9,10] AHX elongated the shoots and roots of bentgrass seedlings, whereas ICA suppressed them dose-dependently.[9,10] Afterward, a metabolite of AHX common in plants, 2-aza-8-oxohypoxanthine (AOH), was found, and AOH also exhibited growth-regulating activity against the bentgrass seedlings

Received: April 7, 2020

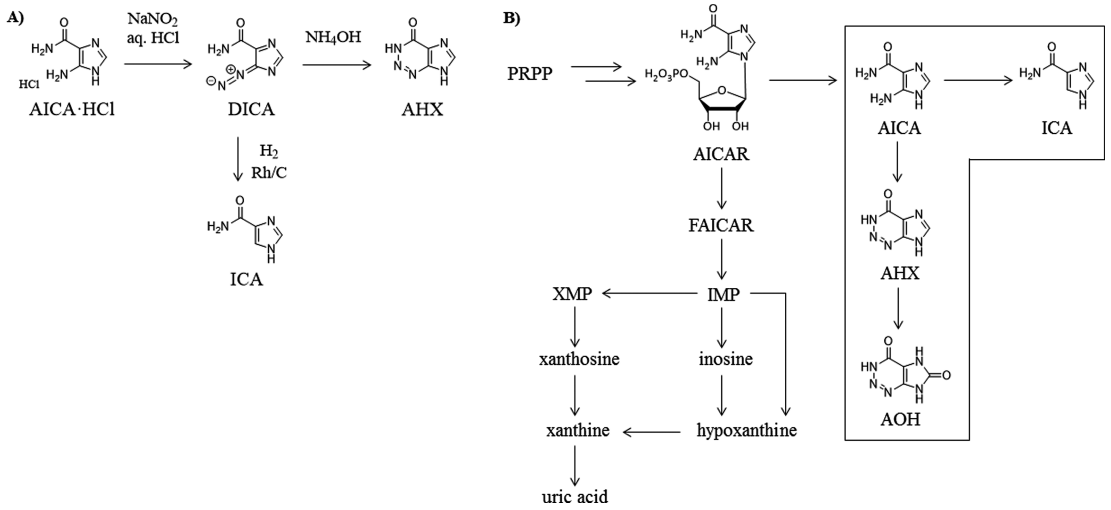


Figure 1. (A) Chemical synthetic route from AICA to AHX and ICA and (B) purine metabolic pathway common in plants, animals, and microorganisms. The two consecutive arrows in (B) represent a multistage enzyme reaction. The black frame represents the novel purine metabolic pathway producing FCs in plants. FAICAR, *N*-formyl-AICAR; XMP, xanthosine monophosphate.

similar to AHX.[11] The three compounds were named "fairy chemicals" (FCs) after the title of the article in *Nature* in 2014 that covered our research.[12] FCs exhibited growth-regulating activity not only against bentgrass but also against various kinds of plants regardless of the families they belong to, and it was also elucidated that FCs were endogenously produced in plants.[9−11,13,14] Furthermore, yields of rice grains, wheat grains, and other crops were significantly increased and plants developed tolerances against cold, hot, and salt stress by treatment with FCs, suggesting the possibility of their practical use in agriculture.[9,10,15,16] As mentioned above, FCs are natural compounds with a variety of beneficial biological activities; however, it is little known about how they are biosynthesized in fungi, especially in the fairy ring-forming fungi.

FCs are chemically synthesized from a common precursor, 5-aminoimidazole-4-carboxamide (AICA) (Figure 1A).[17−20] Previous studies about FCs biosynthesis in plants proved that there was a biosynthetic pathway of FCs similar to the chemical synthetic one and FCs were biosynthesized from AICA from the purine metabolic pathway (Figure 1B).[11,13,14] Furthermore, it was also reported that AHX was biosynthesized from 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR), which is a precursor of fundamental purine metabolites such as adenosine monophosphate (AMP), inosine monophosphate (IMP), and hypoxanthine in the purine metabolic pathway in *L. sordida* mycelia, and AHX biosynthesis was transcriptionally controlled by AICAR.[21]

In most living organisms, purine nucleotides are biosynthesized by a series of enzymatic reactions that result in the formation of IMP, and IMP is further converted into AMP or guanosine monophosphate to satisfy the purine nucleotide requirements in growing cells.[22−24] Moreover, free purine bases can be directly recycled by a salvage pathway in the cells and incorporated into purine nucleotide pools. The reaction is catalyzed by phosphoribosyltransferases, a group of enzymes that are responsible for the transfer of the phosphoribosyl group from α-D-5-phosphoribosyl 1-pyrophosphate (PRPP) onto a nitrogenous base to form an *N*-ribose monophosphate and pyrophosphate.[24] The enzymes are necessary not only for the purine biosynthesis but also for the biosynthesis of pyrimidine, pyridine, histidine, and tryptophan.[24]

Adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) is the key enzyme in the direct recycling of free adenine into purine nucleotide pools and catalyzes the Mg^{2+}-dependent reversible interconversion between adenine and AMP.[22,25−33] In APRT-deficient human cells, it is known that adenine is converted into a poorly soluble derivative, 2,8-dihydroxyadenine (DHA), and the accumulation of DHA leads to urolithiasis and renal failure.[34] APRT also recognizes other substances having the purine-like skeleton such as hypoxanthine and AICA as substrates.[35] In the previous research, a precursor incubation experiment that added AICAR into an *L. sordida* mycelial culture was performed, and the accumulation of AHX was observed after supplementation.[21] In addition, it was also reported that the expression level of the APRT gene was stimulated by addition of AICAR into *L. sordida*.[21]

Herein, we first examined whether FCs endogenously existed in various mushrooms or not. Subsequently, we tried to elucidate the biosynthetic pathway to AHX and ICA in *L. sordida* mycelia which are able to produce large amounts of both of the compounds. In order to elucidate the relationship between AHX and ICA biosynthesis and the purine metabolic pathway in *L. sordida* mycelia, a labeling experiment using ^{13}C-double-labeled Gly ([1,2-$^{13}C_2$]Gly) was performed. Furthermore, the fungus was incubated with ^{13}C-double-labeled AICA to confirm whether AICA was the precursor of AHX and ICA. Finally, an APRT gene was cloned from *L. sordida* mycelia, the gene was overexpressed in *Escherichia coli*, and the enzyme activity of recombinant LsAPRT (rLsAPRT) was tested to investigate the involvement of LsAPRT in the biosynthesis of AHX and ICA.

RESULTS AND DISCUSSION

In previous reviews, we introduced the preliminary result of the endogenous existence of AHX and AOH in 11 species of mushrooms (*Cortinarius caperatus*, *Hypholoma sublateritium*, *Lyophyllum decastes*, *Tricholoma flavoviens*, *Flammulina velutipes*, *L. sordida*, *Lyophyllum connatum*, *Lepista nuda*, *Grifola frondosa*, *Pholiota adiposa*, and *Lyophyllum shimeji*).[13,14] Very recently, we developed an exhaustive and efficient method for

https://dx.doi.org/10.1021/acs.jnatprod.0c00394
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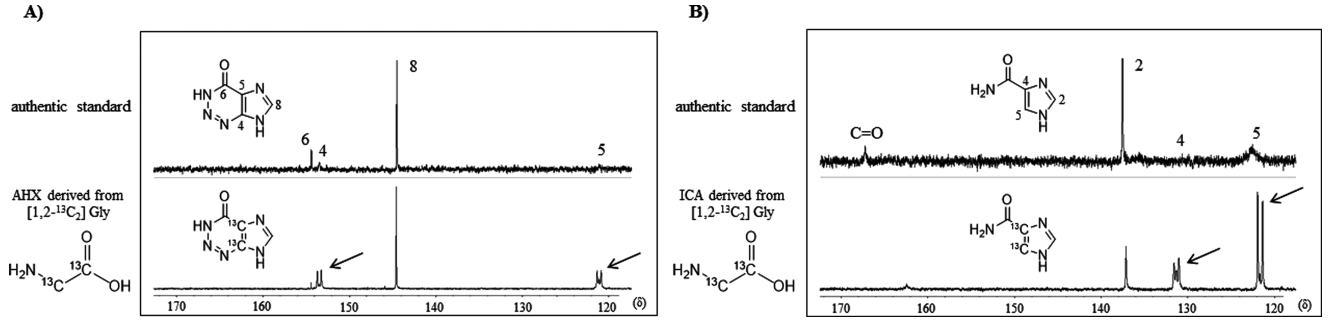

Figure 2. (A) ^{13}C NMR spectra of AHX and (B) ICA derived from [1,2-$^{13}C_2$]Gly in CD_3OD. ^{13}C NMR spectra were recorded on a JEOL lambda-500 spectrometer at 125 MHz. Top and bottom indicate the spectra of authentic standards and purified AHX and ICA derived from labeled Gly, respectively. Black arrows represent the signals of ^{13}C from [1,2-$^{13}C_2$]Gly.

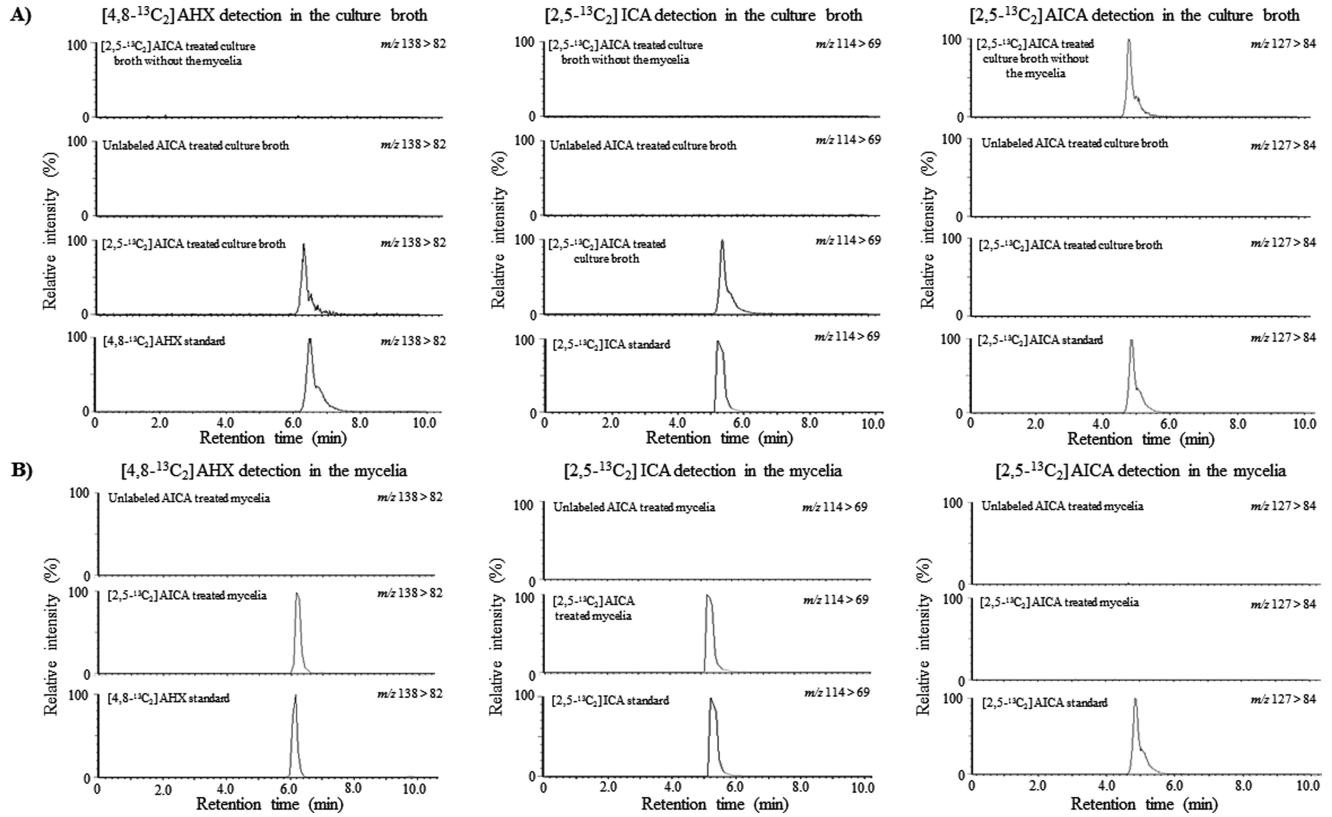

Figure 3. (A) LC-MS/MS spectra of ^{13}C-double-labeled AHX, ICA, and AICA in the culture broth and (B) in the mycelia. The mycelia were extracted after incubation with 0.2 mM unlabeled AICA or labeled AICA for 5 days. Labeled AHX and AICA were detected in the negative mode, and labeled ICA was analyzed in the positive mode. LC-MS/MS chromatograms indicate multiple reaction monitoring (MRM) for [4,8-$^{13}C_2$]AHX, [2,5-$^{13}C_2$]ICA, and [2,5-$^{13}C_2$]AICA, respectively.

detection and quantification of FCs by LC-MS/MS.[36] By this method, ICA detection that had been almost impossible due to its very low sensitivity has become possible. Therefore, we analyzed the extracts of the mushrooms by this method. As a result, the presence of AHX and ICA in various mushrooms and the presence of AOH in only *L. sordida* and *L. nuda* were proven for the first time (Figures S1, S2, S3, Table S1). Furthermore, all FCs in *L. sordida* mycelia were successfully quantified (Figure S4). *L. sordida* mycelia produce large amounts of AHX and do not have a plant-like xanthine oxidase that converts AHX into AOH as judged from genome information.[37] Therefore, AOH had not been detected from *L. sordida* mycelia previously. This is the first detection of AOH from the fungus, although its content was very low. AHX and ICA were endogenously produced in various mushrooms; however, *L. sordida* mycelia in particular produce high amounts of both of the compounds and are cultured easily. Therefore, the fungus was chosen for further investigation of the biosynthetic pathway to the compounds.

As mentioned in the introduction, we have revealed that FCs are biosynthesized from the purine metabolic pathway in plants.[11,13,14] To investigate whether AHX and ICA are produced by a similar biosynthetic pathway in *L. sordida* mycelia, the fungus was incubated with [1,2-$^{13}C_2$]Gly. It is known that two juncture carbons in the purine carbon skeleton of all the purine bases come from Gly in the purine metabolic pathway.[38] After incubation of the fungus with double-labeled Gly, AHX and ICA were purified from the culture broth and

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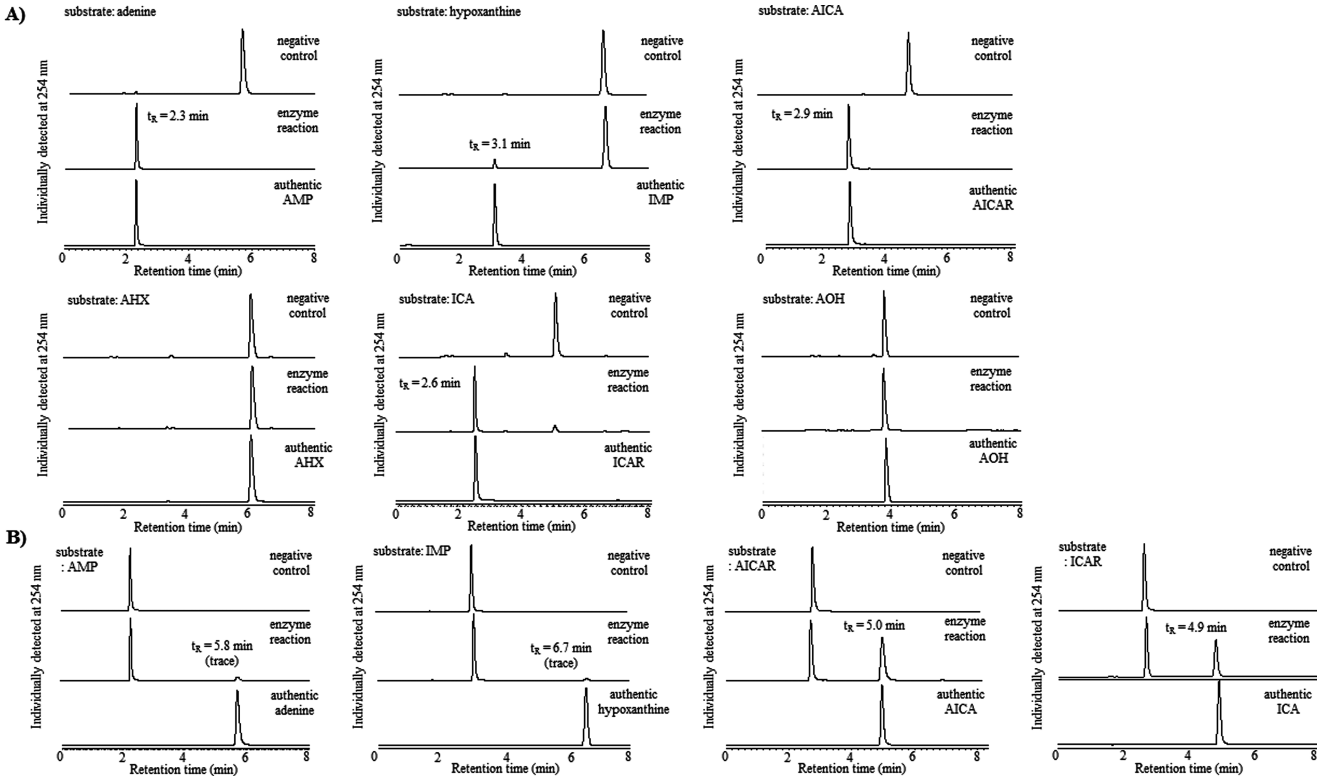

Figure 4. (A) UPLC analysis of the products of phosphoribosylation and (B) dephosphoribosylation using rLsAPRT. Purified rLsAPRT was incubated with each substrate in the presence of Mg^{2+} and PRPP or pyrophosphate at 37 °C for 12 h. Boiled enzyme was used for the negative control. The reaction products were identified by comparing the retention time with that of authentic standards. t_R, retention time.

both of the compounds were analyzed by ^{13}C NMR spectroscopy and LC-MS/MS. In the ^{13}C NMR spectra, the signals of the juncture carbons, C-4 and C-5, in AHX and ICA were remarkably increased and coupled to each other to form doublets (Figure 2). In the LC-MS/MS analyses, unlabeled AHX and ICA (authentic standards) gave only native parent ion peaks (m/z 136 and 110, respectively) in the negative mode (Figure S5A,B). In contrast, purified AHX and ICA gave parent ion peaks in which two carbons were derived from labeled Gly (m/z 138 and 112, respectively) along with native parent ion peaks (Figure S5C,D). These results indicated that two carbons of Gly were incorporated at the junctures of the purine-like structure in AHX and ICA, and both compounds were biosynthesized from the purine metabolic pathway in *L. sordida* mycelia like the other general purine bases.

In the chemical synthesis of AHX and ICA, AICA is the precursor of both of the compounds.[17−20] AICA reacts with $NaNO_2$ to form an unstable intermediate, 4-diazo-4*H*-imidazole-5-carboxamide (DICA), and treatment of DICA with NH_3 produces AHX (Figure 1A). ICA is also synthesized by hydrogenolysis of DICA (Figure 1A). We thought that AHX and ICA are biosynthesized in *L. sordida* mycelia by a route similar to that of the chemical synthesis. In order to examine whether AICA is the precursor of both of the compounds in *L. sordida* mycelia or not, the fungus was incubated with unlabeled AICA. As a result, the amounts of AHX and ICA were significantly increased by the treatment (Figure S6). Subsequently, ^{13}C-double-labeled AICA, AHX, and ICA were synthesized, and the fungus was incubated with labeled AICA (2.0 μmol).[39] An unlabeled AICA treatment group and a labeled AICA treatment group without the fungus were used as the controls in this experiment, and the culture broth and the mycelial extract were analyzed by LC-MS/MS. Labeled AHX and AICA were detected in the negative mode, and labeled ICA was analyzed in the positive mode. As a result, in the culture broth, labeled AHX and labeled ICA were detected only in the labeled AICA treatment group and the amounts of labeled AHX and ICA were 1.22 and 0.37 μmol, respectively (Figure 3A). Residual labeled AICA in the medium after the incubation of the fungus was not detected, while the amount of labeled AICA in the medium without the fungus was 1.97 μmol (Figure 3A), indicating that labeled AICA was stable under this culture condition. In the mycelial extract, the amounts of labeled AHX and ICA were very low (12.8 and 9.0 nmol, respectively), and labeled AICA was not detected (Figure 3B). Summarizing the result, the conversion rates from AICA to AHX and to ICA were 62% and 19%, respectively, and AICA was more used for the biosynthesis of AHX than that of ICA in the fungus. AHX and ICA were for the first time isolated from the culture broth of the fungus, and AICA has never been detected in the culture fluid. The results of the experiment suggested that most of labeled AICA was absorbed into *L. sordida* mycelia, and then the fungus released labeled AHX and ICA as metabolites of labeled AICA out of the cells. This result clearly proved that there was a similar biosynthetic route to AHX and ICA in the fungus to that in plants. The reaction from AICA to AHX requires a nitrogen source. According to the previous study, *L. sordida* mycelia have a gene encoding nitric oxide (NO) synthase (NOS), and NO is a candidate for the nitrogen source of the reaction.[37]

As mentioned above, AHX and ICA were endogenously produced on the pathway as shown in Figure 1B not only in plants but also in *L. sordida* mycelia. In the previous study, an incubation experiment adding AICAR to a *L. sordida* mycelial

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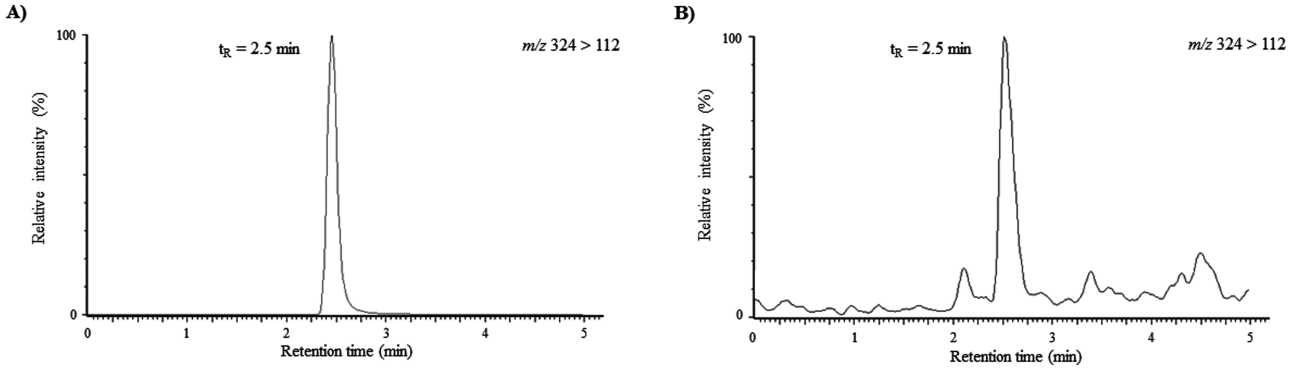

Figure 5. Detection of endogenous ICAR in *L. sordida* mycelia by LC-MS/MS. The freeze-dried mycelia were extracted and the sample was analyzed by LC-MS/MS as described in the Experimental Section. ICAR was detected in the positive mode. LC-MS/MS chromatograms indicate MRM for (A) authentic ICAR and (B) endogenous ICAR in the sample, respectively. t_R, retention time.

culture was performed, and consumption of AICAR and accumulation of AHX were observed after supplementation.[21] Furthermore, quantitative reverse transcription-PCR analysis demonstrated that the APRT gene of *L. sordida* mycelia showed transcriptional enhancement after adding of AICAR.[21] Considering these results, we thought that LsAPRT was involved in the reaction from AICAR to AICA; therefore, the gene was overexpressed in *E. coli*. rLsAPRT purified by Ni affinity column chromatography gave a single band between 20 and 25 kDa in SDS-PAGE, in agreement with the molecular mass, 22.1 kDa, calculated from its amino acid sequence (Figure S7). As a result, rLsAPRT (about 2 to 4 mg) was purified from 1 L of transgenic *E. coli* cell culture and used in the following enzyme assays.

The phosphoribosylation activity of rLsAPRT toward adenine, hypoxanthine, AICA, AHX, ICA, and AOH and its dephosphoribosylation activity using AMP, IMP, AICAR, and ICA-ribotide (ICAR) were tested. Boiled enzyme was used in the negative control. Evaluation of the results was done using ultraperformance liquid chromatography (UPLC) of the reaction mixtures. rLsAPRT transformed the majority of adenine, AICA, and ICA to their phosphoribosylated products AMP, AICAR, and ICAR (Figure 4A). As shown in Figure S8A, the phosphoribosylation conversion efficiency was the highest for adenine. On the other hand, the activity with hypoxanthine was very low, and no product was formed in the reaction employing AHX or AOH as an acceptor (Figure 4A). Furthermore, rLsAPRT also catalyzed the dephosphoribosylation and converted AMP, IMP, AICAR, and ICAR into their corresponding free bases (Figure 4B). However, the activity of the reverse reaction utilizing all the substrates was considerably lower than the phosphoribosylation activity. The dephosphoribosylation activity of the enzyme toward AICAR was stronger than that for ICAR (Figure S8B). rLsAPRT exhibited the highest activity at 40 °C and was stable at temperatures lower than 50 °C (Figure S9A,B). The enzyme showed the highest activity at pH 5.0, and the activity remained more than 60% between pH 5.0 and 10.0 (Figure S9C,D).

In this study, the function of rLsAPRT became clear *in vitro* for the first time. However, contrary to our expectation, the conversion activity from AICAR to AICA was much lower than that from AICA to AICAR. In the previous study, it was reported that the phosphoribosylation activity toward adenine of APRT derived from *Leishmania donovani* was more than 10 times higher than the dephosphoribosylation activity toward AMP.[40] Considering that *L. sordida* mycelia produce large amounts of AHX and ICA, AICA might be quickly metabolized to AHX, ICA, and/or other derivatives, and some compounds and/or proteins might help the dephosphoribosylation activity of LsAPRT *in vivo*. More notably, rLsAPRT recognized ICA but not AHX and AOH as a substrate, and LC-MS/MS indicated the endogenous existence of ICAR in *L. sordida* mycelia (Figure 5). The presence of ICAR in organisms was verified by this result for the first time. A plant hormone, cytokinins, plays vital roles in the regulation of multiple physiological processes in plants, such as cell proliferation, differentiation, leaf senescence, and responses to biotic and abiotic signals.[41−45] It was previously reported that APRT1 in *Arabidopsis* catalyzed the conversion from isopentenyladenine (iP) and *trans*-zeatin (*trans*-Z) to their nucleotides.[46] Deactivation of APRT1 in the plant led to excess accumulation of the two cytokinins, thus evoking various cytokinin-regulated responses such as delayed leaf senescence, anthocyanin accumulation, and downstream gene expression.[46] In addition, cytokinins derived from *Ustilago maydis*, the causative principle of maize smut, play a key role in regulating *U. maydis* and *Zea mays* interaction.[47] The strain in which biosynthesis of cytokinins was deficient elicited fewer and smaller tumors than wild type.[47] iP and *cis*-zeatin (*cis*-Z) are converted into their nucleotides by APRT in *U. maydis*, and the infection to maize might be regulated by phosphoribosylation of them. Therefore, the biological activities of FCs might be regulated by phosphoribosylation and dephosphoribosylation in organisms like in the case of cytokinins. Furthermore, genome sequence analysis of *L. sordida* mycelia showed that the fungus has other genes encoding phosphoribosyltransferases.[37] Generally, the substrate specificity of phosphoribosyltransferases such as APRT and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is different from each other and the enzymes recognize different compounds having a purine-like skeleton as substrates.[48,49] Therefore, AHX and AOH might be metabolized to their ribotides by other phosphoribosyltransferases such as HGPRT in *L. sordida* mycelia.

FCs are plant growth regulators isolated from the fairy ring-forming fungus *L. sordida* as the causative principles of fairy rings. In conclusion, we succeeded in detecting at least one kind of FC in 11 species of mushrooms and partially elucidating the biosynthetic pathway to AHX and ICA in *L. sordida* mycelia. The most important findings in this study were that rLsAPRT catalyzed the reversible interconversion

https://dx.doi.org/10.1021/acs.jnatprod.0c00394
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between ICA and ICAR, and the endogenous existence of ICAR was verified in *L. sordida*.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{13}C NMR spectra were recorded on a JEOL lambda-500 spectrometer at 125 MHz. LC-MS/MS analyses were performed with a UPLC system (Shimadzu) coupled with a tandem LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) and a UPLC system (Nihon Waters) coupled with a tandem Xevo TQ-S micro mass spectrometer (Nihon Waters). HPLC separations were performed with a Jasco Gulliver system (PU-2089 Plus quaternary gradient pump, AS-2010 Plus multiwavelength detector, AS-2055 Plus Intelligent sampler, CO-2060 Plus Intelligent column thermostat, LC-Net II/ADC). The enzyme assays of rLsAPRT were performed with a UPLC system (quaternary solvent manager, sample manager-FTN, PDA eλ detector, Nihon Waters). Stable-isotope-labeled Gly ([1,2-$^{13}C_2$]Gly) and authentic AICAR were purchased from Cambridge Isotope Laboratories and Sigma-Aldrich Japan, respectively. ^{13}C-double-labeled AICA, AHX, ICA, and authentic ICAR were synthesized according to the methods previously described.[39,50] All solvents used throughout the experiments were obtained from Kanto Chemical.

Fungal Material. The fruiting bodies of *Cortinarius caperatus*, *Hypholoma sublateritium*, *Tricholoma flavoviens*, *Lepista sordida*, *Lyophyllum connatum*, *Lepista nuda*, and *Pholiota adiposa* were collected at Narusawa village, Yamanashi Prefecture in Japan. The fruiting bodies of *Grifola frondosa*, *Flammulina velutipes*, *Lyophyllum decastes*, and *Lyophyllum shimeji* were purchased at local supermarkets in Japan.

Detection of Endogenous FCs in Mushrooms. Lyophilized mushrooms (about 50 mg) were used in this experiment. Extraction and fractionation were performed as previously described.[36] The fraction was dissolved in 100 μL of 80% MeCN in 0.05% formic acid (FA) and subjected to LC-MS/MS (Thermo Fisher Scientific). A PC-HILIC column (ϕ 2.0 × 100 mm, Osaka Soda) was used in this analysis (solvent, 95% MeCN in 0.05% FA; column oven, 40 °C; flow rate, 0.2 mL/min for ICA, 0.4 mL/min for AHX and AOH; injection volume, 5 μL). For AHX and AOH detection, MS analysis was performed in the negative mode with the following source parameters: sheath gas flow, 50; auxiliary gas flow, 10; tube lens, −63 V; capillary voltage, −16 V; ion spray voltage, 3 kV. For ICA detection, MS analysis was performed in the positive mode with the following source parameters: sheath gas flow, 50; auxiliary gas flow, 10; tube lens, −14.7 V; capillary voltage, 1 V; ion spray voltage, 3 kV. The compounds were identified by exact mass and characteristic transition (precursor ion to daughter ion).

***Lepista sordida* Strain and Culture Conditions.** The mycelia of *L. sordida* (NBRC112841) were precultured on potato dextrose agar (PDA) medium to obtain actively growing cultures, and the inoculated mycelia were incubated at 25 °C for a month. After growth, the mycelial plate was stored at 4 °C until it was used for the experiments. For a labeling experiment using [1,2-$^{13}C_2$]Gly, 30 pieces (6 mm diameter) cut from four-week-cultured mycelia on PDA medium were placed into each 500 mL Erlenmeyer flask containing 250 mL of YG medium (0.05% yeast extract and 0.5% D-glucose). One milliliter of 62.5 mM [1,2-$^{13}C_2$]Gly filtered with a membrane filter was added to the cultures, and the cultures were incubated for 5 weeks (25 °C, 120 rpm). For total RNA extraction and endogenous FCs and ICAR detection in *L. sordida* mycelia, two pieces (8.5 mm diameter) cut from a similar mycelial plate were inoculated into a 50 mL Erlenmeyer flask containing 10 mL of YG medium (0.3% yeast extract, 1% D-glucose, 0.05% KH_2PO_4, 0.05% Na_2HPO_4 and pH 5.5), and the culture was incubated for 3 weeks (25 °C, 120 rpm). The mycelia were collected by filtration and frozen at −80 °C until further experiments. For a labeling experiment using ^{13}C-double-labeled AICA, 10 pieces (5.0 mm diameter) were inoculated into a 50 mL Erlenmeyer flask containing 10 mL of modified Czapek medium (0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% KCl, 0.002% $CuSO_4{\cdot}5H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$, 0.0001% $ZnSO_4{\cdot}7H_2O$, 0.0001% $MnSO_4{\cdot}5H_2O$, 1% D-glucose, 0.05% KH_2PO_4, 0.05% Na_2HPO_4, pH 5.5), and the culture was incubated for 1 week (25 °C, 120 rpm). Labeled AICA or unlabeled AICA filtered through a membrane filter was added to the culture, and the mycelia were further cultured for 5 days (25 °C, 120 rpm).

Isolation and Detection of Labeled AHX and Labeled ICA Derived from [1,2-$^{13}C_2$]Gly. The culture broth (1 L) of *L. sordida* mycelia was filtered and concentrated under reduced pressure. The concentrate (5.1 g) was fractionated by octa-decyl-silyl gel (Cosmosil 140C18-OPN, Nacalai Tesque) flash column chromatography: 5% (fractions 1 to 5), 10% (fractions 6 to 10), 25% (fractions 11 to 15), and 50% (fractions 16 to 20) MeOH in H_2O and MeOH (fractions 21 to 25) to obtain 25 fractions (fractions 1 to 25). Fractions eluted with 10% MeOH were combined, and the fraction (400.7 mg) was separated by HPLC (column, Develosil C30-UG-5, ϕ 20 × 250 mm, Nomura Chemical; solvent, 2% MeOH in 0.02% trifluoroacetic acid (TFA); flow rate, 5.0 mL/min; UV detection, 210 nm: column, Cosmosil 5-PYE waters, ϕ 10 × 250 mm, Nacalai Tesque; solvent, 2% MeOH in 0.02% TFA; flow rate, 2.0 mL/min; UV detection, 210 nm) to afford AHX (4.9 mg) and ICA (3.5 mg). Purified AHX and ICA were dissolved with 95% MeOH in 0.05% FA. The sample was centrifuged, and then the supernatant was subjected to LC-MS/MS analysis (Thermo Fisher Scientific). A PC-HILIC column was used in this analysis (solvent, 95% MeOH in 0.05% FA; column oven, 40 °C; flow rate, 0.2 mL/min; injection volume, 10 μL). MS analysis including ICA was performed in the negative mode with the same source parameters above.

Heterologous Expression of Recombinant LsAPRT in *E. coli*. *L. sordida* mycelia were frozen in liquid nitrogen and ground into a powder. Total RNA was extracted from the resulting mycelial powder using TRIzol reagent (Thermo Fisher Scientific) and RNeasy mini kit(Qiagen), according to the manufacturer's instruction. cDNA was reverse-transcribed from 500 ng of total RNA with Prime Script RT reagent kit perfect real time (Takara Bio). A full-length cDNA fragment of the LsAPRT gene was amplified by RT-PCR using PrimeSTAR Max DNA polymerase (Takara Bio). A forward primer (5′-gtgcatcatcatcatcatatcgaaggtaggcatATGGACGTTGAGTACAT-TAAAGACCAATTGAC-3′) and a reverse primer (5′-acaagcttgaattcgTCAATCATCCGATTGAACGATCG-3′) were designed based on the nucleotide sequence of the LsAPRT gene.[21] The reaction mixture (10 μL) contained 50 ng of total cDNA, 5 μL of PrimeSTAR Max DNA polymerase, and 0.25 μM of each primer. Cycling conditions were set as follows: preincubation, 1 cycle of 94 °C for 2 min; amplification, 35 cycles of 98 °C for 15 s, 60 °C for 30 s, and 68 °C for 15 s. The amplified PCR products were analyzed by electrophoresis using a 1.2% agarose gel and ligated into pCold I expression vector (Takara Bio) between the *Nde*I and *Bam*HI sites using Gibson assembly master mix (New England BioLabs Japan), followed by transformation into *E. coli* DH5α competent cells (Nippon Gene). The plasmid DNA (pCold I-*LsAPRT*) was extracted with the HiYield plasmid mini kit (RBC Bioscience). The authenticity of the resulting plasmid DNA was confirmed by PCR analysis and sequencing, and then the plasmid was transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells (Agilent Technologies Japan). The cells with the plasmid were grown in 1 L of lysogeny broth (LB) medium containing 100 μg/mL ampicillin at 37 °C until OD_{600} reached 0.4. After incubation at 4 °C for 30 min, rLsAPRT was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were cultured at 15 °C for at least 24 h. The cells were harvested by centrifugation and stored at −80 °C until protein extraction.

Purification of rLsAPRT. The cells were suspended in extraction buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10% glycerol, and 30 mM imidazole). After lysis by sonication for 10 min on ice, the cell debris was removed by centrifugation. The supernatant was filtered using a membrane filter and subjected to Ni affinity column chromatography using HisTrap HP (GE Healthcare Japan). After washing the column with wash buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10% glycerol, and 70 mM imidazole), 6× His-tagged rLsAPRT was eluted with elution buffer (20 mM Tris-HCl, pH 7.4,

https://dx.doi.org/10.1021/acs.jnatprod.0c00394
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500 mM NaCl, 10% glycerol, and 1 M imidazole) with a linear gradient (0.07 to 1 M imidazole). The recombinant protein eluted fractions were confirmed by 15% SDS-PAGE with Coomassie brilliant blue staining and desalted against dialysis buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 10% glycerol) at 4 °C. After dialysis, the protein solution was concentrated and replaced with enzyme buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 20% glycerol) using Amicon Ultra-0.5 mL 10K (Merck). The purified protein concentration was quantified using the Pierce 660 nm protein assay reagent (Thermo Fisher Scientific) with BSA as the standard.

***In Vitro* Enzyme Assays of rLsAPRT.** For the phosphoribosylation activity, an enzyme reaction mixture (100 μL) containing 0.2 mM substrate (adenine, hypoxanthine, AICA, AHX, ICA, or AOH), 1 mM PRPP (Sigma-Aldrich Japan), 10 mM $MgCl_2 \cdot 6H_2O$, 20 mM Tris-HCl (pH 7.4), and 100 μg/mL rLsAPRT was prepared. For the dephosphoribosylation activity, the enzyme reaction mixture (100 μL) consisted of 0.2 mM substrate (AMP, IMP, AICAR or ICAR), 1 mM sodium pyrophosphate decahydrate (Sigma-Aldrich Japan), 10 mM $MgCl_2 \cdot 6H_2O$, 20 mM Tris-HCl (pH 7.4), and 100 μg/mL rLsAPRT. Enzyme was boiled for 10 min before the reaction for use as the negative control. The reaction mixture was preincubated at 37 °C for 5 min, and then the reaction was initiated by adding the enzyme solution. The reaction was terminated by boiling the mixture for 5 min, and then the mixture was centrifuged to precipitate proteins. The optimum temperature and thermal stability of rLsAPRT were tested in 20 mM Tris-HCl (pH 7.4) at different temperatures (5 to 90 °C). The effect of pH was determined in different buffers (pH 2.0 to 3.0, 20 mM Gly-HCl; pH 3.0 to 5.0, 20 mM sodium acetate−acetic acid; pH 5.0 to 7.0, 20 mM PIPES-NaOH; pH 7.0 to 8.0, 20 mM Tris-HCl; pH 8.0 to 10.0, 20 mM Gly-NaOH) at 40 °C. The enzyme reaction products in the supernatant were detected by UPLC analysis (column, CAPCELL PAK ADME S3, ϕ 2.1 × 250 mm, Osaka Soda; solvent, A: 10 mM ammonium formate, B: MeOH, 2% solvent B or a linear gradient (20% to 100% solvent B) for adenine and AMP; flow rate, 0.2 mL/min; column oven, 40 °C).

Detection of Labeled AHX and Labeled ICA Derived from ^{13}C-Double-Labeled AICA and Endogenous ICAR. *L. sordida* mycelia were freeze-dried and extracted according to the high-sensitivity detection method for purine metabolites.[36] The sample was dissolved in 100 μL of H_2O with 0.05% FA and subjected to LC-MS/MS (Nihon Waters). A CAPCELL PAK ADME S3 column was used in this analysis (solvent, A: 10 mM ammonium formate, B: MeOH, 2% solvent B; column oven, 40 °C; flow rate, 0.2 mL/min; injection volume, 1 μL). For AHX detection, MS analysis was performed in the negative mode with the following source parameters: capillary voltage, 2.5 kV; cone voltage, 40 V; collision energy, 12 V. For ICA detection, MS analysis was performed in the positive mode: capillary voltage, 1.0 kV; cone voltage, 10 V; collision energy, 28 V. For AICA detection, MS analysis was performed in the negative mode: capillary voltage, 2.5 kV; cone voltage, 2 V; collision energy, 18 V. For ICAR detection, MS analysis was performed in the positive mode: capillary voltage, 1.0 kV; cone voltage, 4 V; collision energy, 12 V. The following source parameters were common to all the compounds: desolvation temperature, 500 °C; desolvation gas flow, 1000 L/h; cone gas flow, 50 L/h.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00394.

LC-MS/MS spectra of FCs and detection of FCs in mushrooms, quantitative analysis of FCs in *L. sordida* mycelia, MS spectra of ^{13}C-double-labeled AHX and ICA, quantitative analysis of AHX and ICA in *L. sordida* mycelia treated with AICA, SDS-PAGE of rLsAPRT, enzyme properties of rLsAPRT (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Hirokazu Kawagishi − *Graduate School of Science and Technology, Graduate School of Integrated Science and Technology, and Research Institute of Green Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan;* orcid.org/0000-0001-5782-4981; Phone: +81-54-238-4885; Email: kawagishi.hirokazu@shizuoka.ac.jp

Authors

Akinobu Ito − *Graduate School of Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan; Research Fellow of Japan Society for the Promotion of Science, Tokyo 102-0083, Japan*

Jae-Hoon Choi − *Graduate School of Science and Technology, Graduate School of Integrated Science and Technology, and Research Institute of Green Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan*

Hirohide Takemura − *Graduate School of Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan; Research Fellow of Japan Society for the Promotion of Science, Tokyo 102-0083, Japan*

Mihaya Kotajima − *Graduate School of Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan*

Jing Wu − *Research Institute of Green Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan*

Shinji Tokuyama − *Graduate School of Integrated Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan*

Hirofumi Hirai − *Graduate School of Science and Technology, Graduate School of Integrated Science and Technology, and Research Institute of Green Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan*

Tomohiro Asakawa − *Marine Science and Technology, Tokai University, Hiratsuka City, Kanagawa 259-1292, Japan*

Hitoshi Ouchi − *Department of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan*

Makoto Inai − *Department of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan;* orcid.org/0000-0001-7074-0520

Toshiyuki Kan − *Department of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan;* orcid.org/0000-0002-9709-6365

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jnatprod.0c00394

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. K. Okamoto (Ushio Chemix, Shizuoka, Japan) for providing authentic AHX and ICA. This work was partially supported by a Grant-in Aid for Scientific Research on Innovative Areas "Frontier Research on Chemical Communications" (JP17H06402) from MEXT to H.K. and a Japan Society for the Promotion of Science KAKENHI (No. 18J21506) to A.I.

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J. Nat. Prod. XXXX, XXX, XXX−XXX