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Ribosides and Ribotide of a Fairy Chemical, Imidazole-4-carboxamide, as Its Metabolites in Rice

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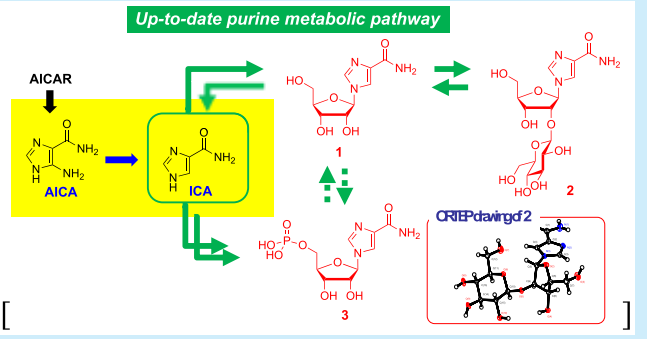
Supporting Information

ABSTRACT: The metabolism of imidazole-4-carboxamide (ICA) in plants has been unknown. Two metabolites (**1** and **2**) were isolated from ICA-treated rice, and their structures were determined by spectroscopic analysis including the single-crystal X-ray diffraction technique and synthesis. The ribotide of ICA (**3**), whose existence was predicted, was also synthesized and detected from the treated rice by LC−MS/MS. These results indicated that rice might interconvert ICA, **1**, and **3** to regulate the biological activity.



Fairy rings are zones of promoted or suppressed grass growth due to the interaction between a fungus and a plant.[1] Since the first scientific article about fairy rings in 1675 and subsequent studies reviewed in *Nature* in 1884,[2] this phenomenon had been attributed to unknown "fairies" before our studies. Our work has disclosed that the fairies, the plant-growth promoter and suppresser produced by a fairy-ring-forming fungus *Lepista sordida*, are 2-azahypoxanthine (AHX) and imidazole-4-carboxamide (ICA), respectively.[3] After the two compounds were found, 2-aza-8-oxohypoxanthine (AOH) was isolated as a common metabolite of AHX in plants, and this compound also stimulated plant growth like AHX.[4] The three compounds were named fairy chemicals (FCs) after the title of the article in *Nature* that prefaced our study.[5] We have also reported the endogenous existence of FCs in plants and the discovery of new routes in the purine metabolic pathway in which FCs are biosynthesized.[4,6] These results indicated that FCs are probably common members of the purine metabolic pathway in plants and microorganisms.[4,6−8]

FCs exhibit growth regulatory activity against all of the plants tested regardless of their belonging families and conferred tolerance to various and continuous stress (low or high temperature, salt, drought, etc.) on the plants.[3,4] Furthermore, the yields of rice, wheat, or other crops were increased by the treatment with each FC in a greenhouse or in field experiments, suggesting the possibility of FC applications in agriculture.[3,4,9] All of the above-mentioned results allowed us to conclude that FCs are biosynthesized on the novel purine metabolic pathway in plants. We have formulated a hypothesis that FCs are a new family of plant hormones, and the elucidation of metabolic pathway of FCs is critical to prove the hypothesis.[8,10,11] Recently, *N*-glucosides of AHX and AOH were discovered as their metabolites in rice.[12] However, the metabolism of ICA in plants has not been revealed. Herein we describe the discovery of three metabolites of ICA in rice (Figure 1).

First of all, to know the metabolism of ICA in plants, rice, cucumber, tomato, lettuce, and komatsuna (*Brassica rapa* L. cv. Rakuten) were incubated with ICA. High-Performance Liquid Chromatography (HPLC) analysis with photodiode array (PDA) detection of the ICA-treated rice indicated that ICA was converted into some metabolites, mainly compounds **1** and **2** (Figure S1a). The conversion of ICA to **1** was also observed in the other plants tested (Figure S1b−e).

Therefore, we tried to isolate the metabolites from rice. Rice was cultivated in ICA-containing culture broth for 2 weeks; then, the rice was divided into roots and shoots. EtOH extracts of the roots were fractionated by octadecyl-silica (ODS) flash

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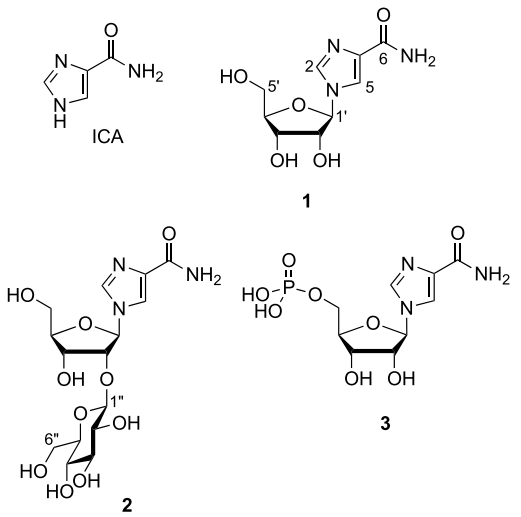

Figure 1. Structures of ICA and its metabolites.

column chromatography, followed by HPLC to yield compounds **1** and **2**.

Compound **1** was purified as a pale-yellow, amorphous material. The molecular formula was determined as $C_9H_{13}N_3O_5$ by high-resolution electrospray ionization mass spectrometry (HRESIMS) (m/z 266.0724 $[M + Na]^+$; calcd for $C_9H_{13}N_3NaO_5$, 266.0753), indicating the presence of five degrees of unsaturation in the molecule. The structure of **1** was elucidated by the interpretation of nuclear magnetic resonance (NMR) spectra including distortionless enhancement by polarization transfer (DEPT), double-quantum-filtered correlation spectroscopy (DQF-COSY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC). The DEPT experiment indicated the presence of one methylene, six methines, and two quaternary carbon atoms. ^{13}C NMR data (δ_C 122.1, 137.5, 138.1, 167.2) indicated that **1** had the same skeleton as ICA.[3b] However, this compound possessed five more carbon atoms than ICA. As the skeleton of the additional moiety, a pentose (C-1′ to C-5′), was constructed by the characteristic ^{13}C NMR chemical shifts at δ_C 62.8, 72.2, 77.7, 87.4, and 92.0, the DQF-COSY correlations (H-1′/H-2′; H-2′/H-3′; H-3′/H-4′; H-4′/H-5′), and the HMBC correlations (H-1′/C-2′; H-2′/C-1′; H-3′/C-1′; H-4′/C-3′; H-5′/C-3′, C-4′) (Figure 2). The NMR data of the pentose moiety in **1** were very similar to those of ribose. The linkage position between the aglycon and ribose

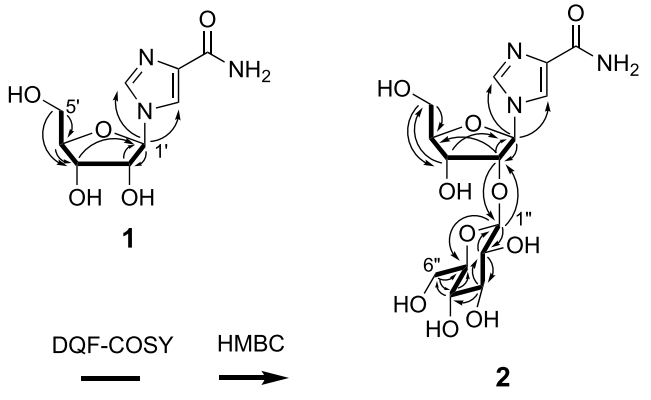

Figure 2. DQF-COSY and HMBC correlations of **1** and **2**.

was determined from the HMBC cross-peak (H-1′/C-2, C-5). All of the data of the isolated **1** and the synthetic one, including specific optical rotation values (isolated **1**, $[\alpha]_D^{25}$ −39 (c 0.28 MeOH); synthetic **1**, $[\alpha]_D^{26}$ −41 (c 0.28 MeOH)) and NMR data (Figures S3 and S4), were identical to each other, indicating that the sugar part of **1** was D-ribose. Although **1** has been synthesized and its antiviral activity against HV/1, RV/13, and PIV/3 and inhibitory activity against nucleotide biosynthesis and adenosine deaminase have been reported, this is the first isolation of the compound from a natural source.[13] The complete assignment of all of the proton and carbon signals of NMR was accomplished as shown in Table 1.

Compound **2** was purified as a pale-yellow, amorphous material. The molecular formula was determined as $C_{15}H_{23}N_3O_{10}$ by HRESIMS (m/z 428.1281 $[M + Na]^+$; calcd for $C_{15}H_{23}N_3NaO_{10}$, 428.1281), indicating the presence of six degrees of unsaturation in the molecule. The structure of **2** was elucidated by the interpretation of NMR data including DEPT, DQF-COSY, HMQC, and HMBC (Figures S5−S8). The DEPT experiment indicated the presence of 2 methylenes, 11 methines, and 2 quaternary carbon atoms. The molecular formula, the unsaturation degrees, the ^{13}C NMR data (δ_C 60.7, 69.4, 83.0, 86.0, 90.7 121.0, 129.5, 136.4, 161.6), and the DEPT data indicated that **2** was a kind of ICA−riboside like **1**. The skeleton of the riboside part (C-2 to C-6; C-1′ to C-5′) was constructed by the DQF-COSY correlations (H-1′/H-2′; H-2′/H-3′; H-3′/H-4′; H-4′/H-5′) (Figure 2 and Figure S7) and the HMBC correlations (H-1′/C-2, C-5, C-2′; H-2′/C-1′, C-4′; H-3′/C-1′,C-5′; H-5′/C-3′,C-4′) (Figure 2 and Figure S8). However, this compound possessed an additional six carbon atoms compared with **1**. The presence of glucose (C-1″ to C-6″) was suggested by the characteristic ^{13}C NMR chemical shifts at δ_C 60.3, 69.2, 73.1, 75.4, 76.0, and 103.0, ^1H NMR chemical shifts, and coupling constants: δ_H 3.22 (H-5″, m), 3.25 (H-2″, m), 3.27 (H-4″, m), 3.36 (H-3″, dd, J = 9.2, 9.2 Hz), 3.51 (H-6″a, dd, J = 12.0, 4.0 Hz), 3.53 (H-6″b, dd, J = 12.0, 3.5 Hz), and 4.46 (H-1″, d, J = 8.0 Hz). The HMBC cross-peaks (H-1″/C-2′, H-2′/C-1″) and the coupling constant of the anomeric proton (J = 8.0 Hz) indicated that the glucose was connected to the ribose at C-2′ via a β-glucosidic bond. The relative configuration of **2** was confirmed by single-crystal X-ray analysis (Figure 3). **2** was hydrolyzed with β-glucosidase to give **1**, indicating that the sugars in **2** were D-ribose and D-glucose (Figure S9). Compound **2** was a novel compound. The complete assignment of all of the proton and carbon signals of NMR was accomplished as shown in Table 1.

Members of a plant hormone family, cytokinins, are interconvertible to their ribosides and ribotides. Hence, we postulated that it was possible that ICA−ribotide (**3**) was also produced by plants. Therefore, to confirm the existence of **1** and **3** in rice, chemical synthesis was investigated. The synthesis of **1** and **3** was commenced with protected AICA−riboside (**4**) (Scheme 1), which was readily obtained from the commercially available inosine.[14] After the removal of the dinitrophenyl group of **4**, the deamination reaction was carried out by the treatment with sodium nitrite under acidic conditions to provide the protected ICA−riboside (**5**). Although utilizing a similar condition as the dinitrophenyl-protected derivative, the diazonium salt formation and subsequent cyclization to the triazine ring proceeded,[14] and deamination occurred in this case. Subsequently, the treatment

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Table 1. ^{1}H and ^{13}C NMR Data for 1 and 2

	1 (in D_2O)		2 (in D_2O)	
	^{1}H	^{13}C	^{1}H	^{13}C
position	δ_H (mult, J, Hz)	δ_C, type	δ_H (mult, J in Hz)	δ_C, type
2	7.93 (d, 1.4)	138.1 CH	8.75 (s)	136.4 CH
4		137.5 C		129.5 C
5	7.92 (d, 1.4)	122.1 CH	8.12 (s)	121.0 CH
6		167.2 C		161.6 C
1′	5.67 (d, 5.5)	92.0 CH	6.01 (d, 4.6)	90.7 CH
2′	4.26 (dd, 5.5, 5.5)	77.7 CH	4.55 (dd, 4.6, 5.2)	83.0 CH
3′	4.20 (dd, 5.5, 3.4)	72.2 CH	4.36 (dd, 5.2, 4.6)	69.4 CH
4′	4.07 (ddd, 3.4, 3.4, 3.5)	87.4 C	4.18 (ddd, 4.6, 4.0, 3.2)	86.0 CH
5′a	3.71 (dd, 12.2, 3.4)	62.8 CH_2	3.70 (dd, 12.8, 4.0)	60.7 CH_2
5′b	3.79 (dd, 12.2, 3.5)		3.79 (dd, 12.8, 3.2)	
1″			4.46 (d, 8.0)	103.0 CH
2″			3.25 (m)	73.1 CH
3″			3.36 (dd, 9.2, 9.2)	75.4 CH
4″			3.27 (m)	69.2 CH
5″			3.22 (m)	76.0 CH
6″a			3.51 (dd, 12.0, 4.0)	60.3 CH_2
6″b			3.53 (dd, 12.0, 3.5)	

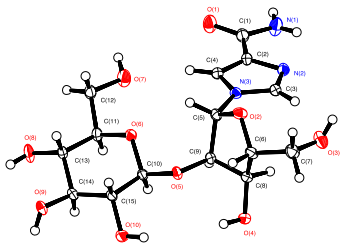
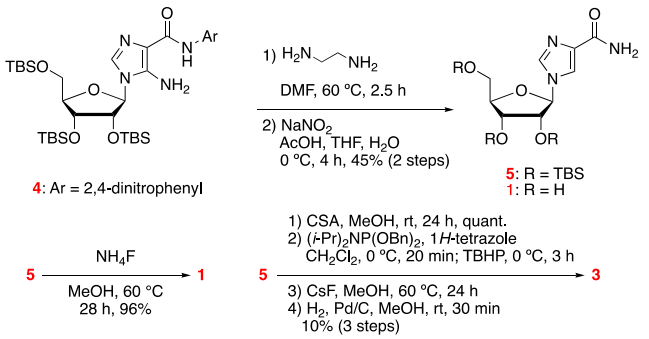


Figure 3. ORTEP drawing of **2**. The structure was solved by a direct method, SHELXL-97 (1), and refined using the SHELXL-97 tool. Crystallographic data have been deposited at The Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 1531773.

Scheme 1. Synthesis of ICA−Riboside (1) and ICA−Ribotide (3)



of **5** with NH_4F to remove the *tert*-butyldimethylsilyl (TBS) groups provided **1**. On the contrary, the synthesis of **3** was demonstrated by utilizing **5**. After the selective removal of the TBS group on the primary alcohol, the incorporation of phosphate ester was carried out by the phosphoramidite method[15] and stepwise deprotection of the TBS and benzyl groups to give **3** (Figure S10).

In the LC−MS/MS analysis using the synthetic **3** as the authentic standard, this compound was detected in the extracts of the ICA-treated rice (Figure S11). ICA exists endogenously and is biosynthesized in plants like AHX and AOH.[3] The existence of **1**−**3** in intact rice was also examined by LC−MS/MS. As a result, **1** and **3** were detected in the extracts of intact rice, indicating the endogenous existence of the compounds in rice (Figure S12). In general, each member on the purine pathway is metabolized to other ones as a ribotide; therefore, ribotides are important intermediates of bioactive free bases on their biosynthetic pathways. ICA may be further metabolized to unknown base(s) via its ribotide **3** in plants.

To examine the metabolism of **1** and **2**, rice was treated with **1** or **2**. Conversions from **1** to ICA and **2** and from **2** to ICA and **1** were observed in both the shoots and roots, suggesting that the interconversion among ICA, **1**, and **2** was reversible in rice (Figure 4 and Figure S13). Adenosine kinases have revealed the predominant activity responsible for phosphorylation for AICA−riboside in the eukaryotic cells.[16] In general, purine nucleotides are converted to their nucleosides by 5′-nucleotidase. The interconversion of **1** and **3** may react with the above enzymes in plants.

The plant growth regulatory activity of **1** and **2** was examined using rice. Both of the compounds showed inhibition activity against the shoots only at high concentration (0.1 mM) and showed no significant effect on the roots (Figure S14). As previously mentioned, cytokinins are interconvertible to their ribosides and ribotides, and those glycosides are the inactive forms of the corresponding free base forms.[17] The free base forms are usually more active than the corresponding ribosides and ribotides in various bioassays, which may be related to rapid uptake and high intrinsic activity.[18] The inhibitory activity of **1** and **2** might be due to ICA that was converted from **1** and **2** in rice (Figure 4 and Figures S13 and S14). Many enzymes involved in cytokinin biosynthesis, interconversion, inactivation, and degradation have been identified and play very important roles in the regulation of endogenous cytokinin homeostasis. The above results suggest that the interconversion among ICA, **1**, and **2** regulates the homeostasis of ICA in rice.

In conclusion, we discovered three metabolites of ICA, ribosides (**1** and **2**) and ribotide (**3**), from ICA-treated rice, and the endogenous existence of **1** and **3** in rice was proved.

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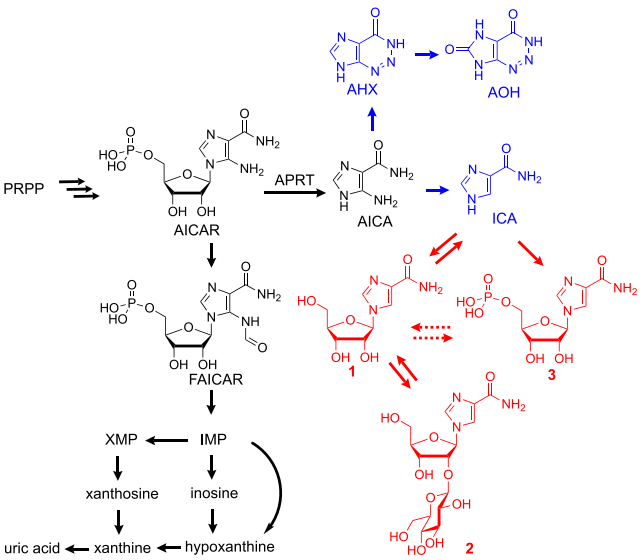

Figure 4. Novel purine metabolic pathway in rice. The route indicated by black arrows was adapted from the KEGG (Kyoto Encyclopedia of Genes and Genomes). The blue arrows and structures show the pathway and metabolites that were found in the previous study. The red arrows show the novel pathway found in this study. The red dashed arrow indicates the predicted pathway. The compounds whose endogenous existence was proven in this study are described in red. AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, AICA: 5-aminoimidazole-4-carboxamide, AHX: 2-azahypoxanthine, AOH: 2-aza-8-oxohypoxanthine, ICA: imidazole-4-carboxamide, APRT: adenine/5-aminoimidazole-4-carboxamide phosphoribosyltransferase, PRPP: phosphoribosyl diphosphate, FAICAR: 5-formAICAR, IMP: inosine monophosphate, XMP: xanthine monophosphate.

The ribosides exhibited weaker inhibition activity than ICA. These results suggest that three metabolites might play an important role in the regulation of exogenous ICA in plants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b02833.

Experimental section, synthesis, HPLC profiles, NMR spectra, plant growth regulatory effect, metabolism, and endogenous existence (PDF)

Accession Codes

CCDC 1531773 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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