Iron Transport

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Mugineic Acid Derivatives as Molecular Probes for the Mechanistic Elucidation of Iron Acquisition in Barley**

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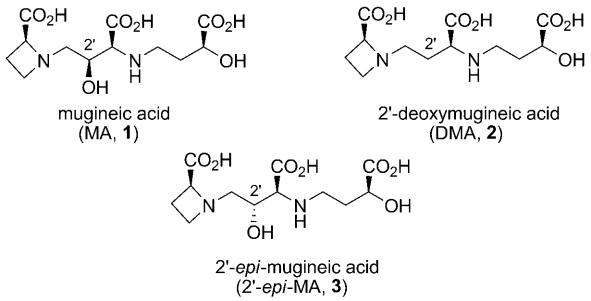
Iron, an essential element for plants, plays versatile and significant roles in a variety of processes, including respiration, photosynthesis, and nitrogen fixation. The element is also indispensable for animals, for whom the source is usually uptake from dietary plants.[1] Thus, iron uptake from the soil by plants is crucial for all living creatures. However, despite the high levels of iron on the surface of the earth,[2] most plants have difficulty absorbing iron in alkaline environments owing to the poor water solubility of its trivalent (Fe^{3+}) salts.[3] To overcome this problem, graminaceous plants have developed a specific strategy based on the secretion of phytosiderophores as chelators to solubilize Fe^{3+} and the uptake of the resulting iron complexes through selective transporters.[4]

Mugineic acid (MA, **1**; Scheme 1) was first identified as a phytosiderophore in barley,[5,6] and analogues of MA have since been isolated from various graminaceous species and cultivars.[7] MA and its analogues all form water-soluble 1:1 complexes with Fe^{III}. In a previous study, we identified a gene that specifically encodes an Fe^{III}·MA transporter (HvYS1) in barley;[8] the gene belongs to the YSL family.[9] The localization and substrate specificity of HvYS1 indicate that it is a specific transporter for the Fe^{III}·MA complex in barley roots.[8] We further revealed that the sixth outer-membrane loop determines the Fe^{III}–phytosiderophore specificity of HvYS1.[10] Therefore, more detailed mechanisms, including the elucidation of the 3D structural pattern of the transporter, the recognition mechanism of Fe^{III} complexes, and the fate of the complexes inside the plants, have become our next focus.



Scheme 1. Structures of mugineic acid (**1**), 2′-deoxymugineic acid (**2**), and 2′-*epi*-mugineic acid (**3**).

To drive these functional studies forward, we had to establish efficient preparative methods for MA derivatives to be utilized as molecular probes. The introduction of functionalities for labeling of the MA skeleton had so far been unsuccessful, mainly because all previously prepared labeled products lost their ability to form Fe^{III} complexes as a result of the structural modifications. We have now established an efficient short-step synthesis of MA (**1**) and 2′-deoxymugineic acid (DMA, **2**),[11] which is a phytosiderophore for rice, wheat, and maize[12] with a similar iron(III)-transport function. Comparison of the activity of synthetic DMA (**2**) with the activities of MA (**1**) and its diastereomer 2′-*epi*-mugineic acid (2′-*epi*-MA, **3**), which was synthesized in a similar manner, clearly showed that these three phytosiderophores exhibit the same level of iron-transport ability.[11] This result provided the clue that the 2′-hydroxy group could be suitable for the labeling of mugineic acid analogues for their functional study. Thus, we introduced various labeling groups at the 2′-hydroxy group of MA (**1**) and investigated the iron-transport activities of the resulting probes.

Because of the multifunctional polar structures of unprotected MA (**1**) or 2′-*epi*-MA (**3**), the preparation of labeled probes by the selective introduction of any substituent at the 2′-hydroxy group is by no means advantageous, even though **1** and **3** can be readily prepared.[11,13] We therefore attempted to synthesize protected MAs with a free 2′-hydroxy group as a labeling precursor. We began the synthesis with Cbz-protected 2-hydroxy-L-allylglycine *tert*-butyl ester **4** (Scheme 2).[11] A 4:1 diastereomeric mixture (in favor of the diastereomer with the nonnatural α configuration of the hydroxy group) was used as obtained by allylic oxidation of Cbz-protected L-allylglycine *tert*-butyl ester. We knew that

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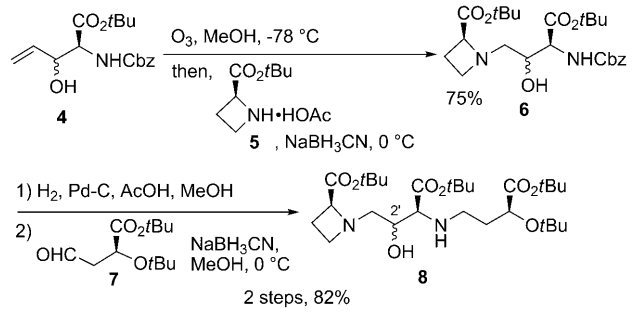
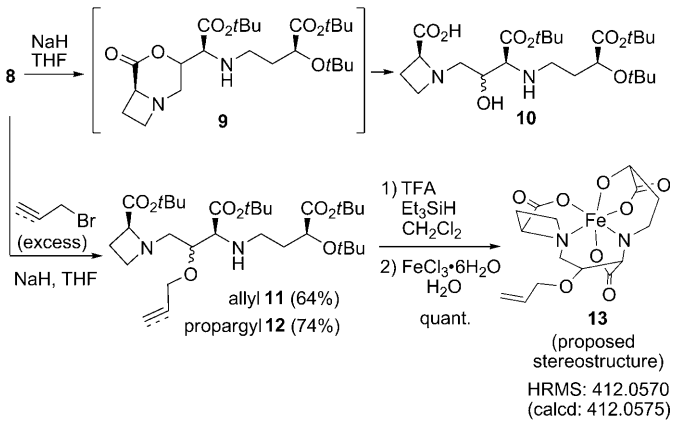


Scheme 2. Synthesis of the protected mugineic acid **8**. Cbz = carbobenzyloxy.

the configuration of the carbon atom bearing the hydroxy group does not affect the iron-transport activity of the product. After the ozonolysis of **4** in methanol at −78 °C, the reaction mixture was treated directly with $NaBH_3CN$ and L-azetidine-2-carboxylic acid *tert*-butyl ester acetic acid salt (**5**) and warmed to 0 °C for 18 h to give **6** in 75 % yield. Hydrogenolytic deprotection of **6** and subsequent direct reductive amination with aldehyde **7** after removal of the palladium catalyst resulted in **8**. Thus, the desired protected MA derivative **8** was obtained in good yield in just three steps from **4**.

We next examined the introduction of various substituents at the 2′-hydroxy group. Acylation of the hydroxy group gave a mixture of O-acylated and N-acylated products. The N-acylated product was generated by migration of the acyl group to the neighboring secondary nitrogen atom. Alkylation of the 2′-hydroxy group was also attempted.[14] The treatment of **8** with sodium hydride to generate an alkoxide anion afforded carboxylic acid **10** through the unexpected formation of lactone **9** and subsequent hydrolysis (Scheme 3). This hydrolytic pathway was a serious problem: even the sterically bulky *tert*-butyl ester did not block lactonization by the alkoxide anion. Thus, the alkylation was conducted by treatment with sodium hydride in the presence of an excess amount of various halides. With almost all alkyl and allyl halides, only carboxylic acid **10** was formed; however, alkylation with the small and reactive reagents, allyl bromide



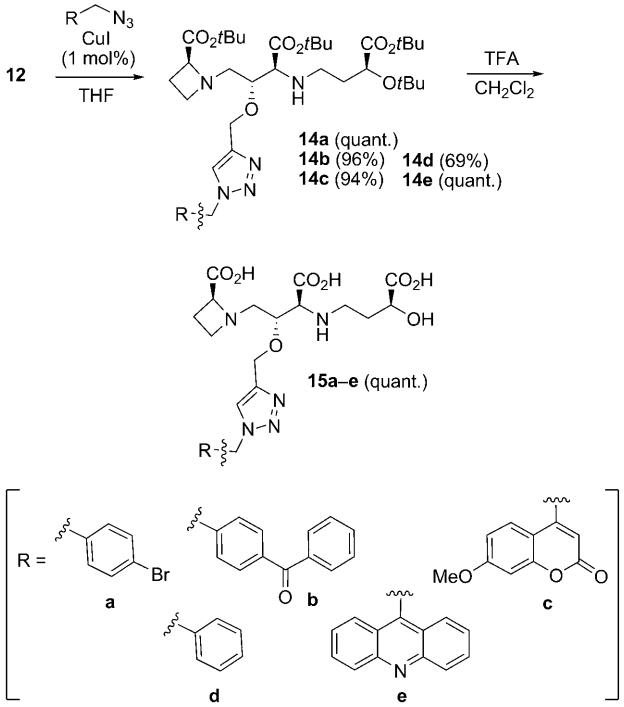
Scheme 3. Synthesis of 2′-O-substituted mugineic acid analogues and the Fe^{III} complex **13**. TFA = trifluoroacetic acid.

and propargyl bromide, proceeded faster than lactonization, and the desired allyl- and propargyl-substituted products **11** and **12** were obtained in 64 and 74 % yield, respectively. With the first 2′-substituted MA analogues in hand, simple 2′-*O*-allylmugineic acid was used to confirm that the substituent on the 2′-hydroxy group does not inhibit complexation with Fe^{III}. Thus, acidic deprotection of **11** followed by treatment with $FeCl_3 \cdot 6H_2O$ in H_2O gave a yellow solution, which suggested the formation of the Fe^{III} complex **13**. The formation of a 1:1 complex **13** was confirmed unambiguously by negative ESI Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS): peaks at the expected mass numbers and the characteristic isotopic pattern of an Fe^{III} complex were observed in the mass spectra (see the Supporting Information).

The formation of complex **13** encouraged us to introduce labeling groups by using the allyl group in **11** as a tether. However, various reactions of **11**, such as metathesis and the Heck reaction, failed to incorporate the desired functional groups, and only the starting compound **11** was recovered. As MA is a strong metal chelator, we assume that **11** forms complexes with various transition metals used as catalysts and inhibits the desired reactions. Alternative transformations of the allyl group, such as hydroboration, dihydroxylation, and ozonolysis, were also prevented by other functional groups, and the desired products were not obtained. We therefore attempted to introduce the desired labeling groups by the use of the propargyl group in **12** as a tether.

Since the C2′ diastereomers were separable at this stage, the major isomer of **12** with the nonnatural α configuration was used for further transformations after separation by column chromatography. A click reaction of **12** with *p*-bromobenzyl azide in the presence of CuI (1 mol %) afforded **14a** in quantitative yield (Scheme 4). This result was a clear contrast to our previous unsuccessful attempts, in which the mugineic acid derivatives inhibited many transition-metal-catalyzed reactions. Although the mechanistic details are not yet understood, we found that a 1:1 complex of **14a** with copper(I) is an excellent catalyst for the click reaction.[15] Finally, the deprotection of **14a** gave the 2′-*p*-bromobenzyltriazole mugineic acid derivative **15a** in quantitative yield. We also prepared the benzophenone derivative **15b** as a photoaffinity probe and the coumarin derivative **15c** as a fluorescent probe in a similar manner. The complexation of **15a**–**15c** with Fe^{III} was confirmed as described for **13** (see the Supporting Information). With the aid of the efficient click reaction, further labeled derivatives were also synthesized readily: the benzyl derivative **15d** was synthesized as an HPLC probe and the acridine derivative **15e** as another fluorescent probe.

We next examined the iron-transport activity of the 2′-O-modified derivatives by measuring the currents associated with electrophysiological transport in *Xenopus laevis* oocytes by modification of a previously reported protocol (see the Supporting Information).[8,16] Oocytes injected with *HvYS1* cRNA exhibited iron-transport ability, whereas those injected with water (negative control) hardly responded to the iron complex **13** or iron complexes of the functionally labeled MA derivatives **15a**–**15c** (Figure 1). The difference in



Scheme 4. Synthesis of labeled mugineic acid derivatives **15**.

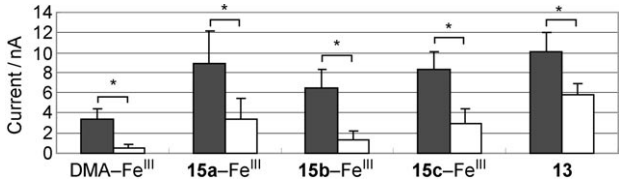


Figure 1. Iron(III)-transport activities of synthetic labeled mugineic acid derivatives as measured by two-electrode voltage-clamp analysis in *Xenopus* oocytes. Currents induced by Fe^{III} complexes of the MA derivatives **15a**, **15b**, and **15c** and the Fe^{III} complex **13** (each 50 μM) in oocytes injected with *HvYS1* cRNA (black bars, $n = 5$–11) or water (white bars, $n = 3$–10). Significant differences between injection with *HvYS1* cRNA and water, as determined by the Tukey test, are indicated with an asterisk (*, $p < 0.01$).

the current intensities between the oocytes injected with *HvYS1* cRNA and those injected with water was remarkable for all Fe^{III} complexes of the 2′-O-modified MA derivatives tested. We therefore conclude that the HvYS1 transporter responded to all of these complexes. Thus, the 2′-OH group appeared to be an appropriate site for the labeling of MA with various groups without the loss of its functions as a phytosiderophore.[17]

We expected to be able to detect the incorporation of the functionally active fluorescence-labeled MA derivative **15c** into *Xenopus* oocytes through the HvYS1 transporter by microscopic observation (see the Supporting Information). The inside of HvYS1-expressed oocyte cells was clearly visualized in the fluorescence mode (Figure 2d,f) with a confocal microscope, whereas the inside of the control oocytes was not (Figure 2b). This experiment indicated that the Fe^{III} complex of the MA analogue **15c** was incorporated

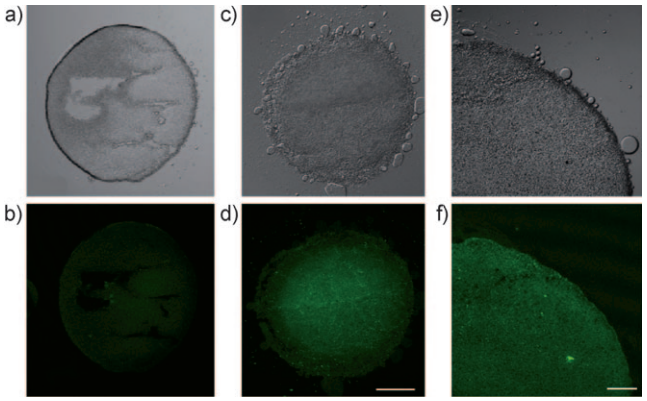


Figure 2. Observation of fluorescence-labeled MA as its Fe^{III} complex in *Xenopus* oocytes. Oocytes injected with a,b) water as a control or c–f) *HvYS1* cRNA were incubated in ND96 buffer containing **15c**–Fe^{III} (50 μM) for 15 min at 16 °C. Uptake of fluorescence-labeled MA–Fe^{III} was monitored by using a laser scanning confocal imaging system (Olympus Fluoview 1000) in a differential interference contrast (DIC) mode (a, c, and e) or in a laser fluorescence mode (b, d, and f). Scale bar: 200 μm (a–d); 50 μm (e and f).

through the HvYS1 transporter. To our knowledge, this is the first direct experimental evidence of the transporter-mediated internalization of mugineic acid into cells.

In this study, an efficient and versatile method was established for the preparation of labeled mugineic acid (MA) derivatives, which form water-soluble Fe^{III} complexes similar to that of natural MA. The complexes were incorporated into cells through the HvYS1 transporter, as observed directly by fluorescence microscopy. We are now investigating the conversion of synthetic 2′-*O*-allylmugineic acid into a tritium-labeled MA derivative through hydrogenation in tritium gas. This compound and the functionally labeled MA derivatives described herein will enable further, more detailed investigation of the molecular mechanism of iron acquisition from roots and the fate of the Fe^{III} complexes of MA and its analogues inside plants.

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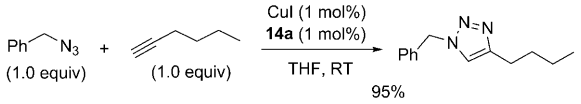
[15] The click reaction of benzyl azide and 1-hexyne in the presence of CuI (1 mol%) and **14a** (1 mol%) afforded the desired 1,2,3-triazole in 95% yield. In contrast, treatment with only CuI (1 mol%) in the absence of **14a** gave the 1,2,3-triazole in less than 20% yield, and the starting materials were recovered.



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