Iron Acquisition

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A Practical Synthesis of the Phytosiderophore 2′-Deoxymugineic Acid: A Key to the Mechanistic Study of Iron Acquisition by Graminaceous Plants**

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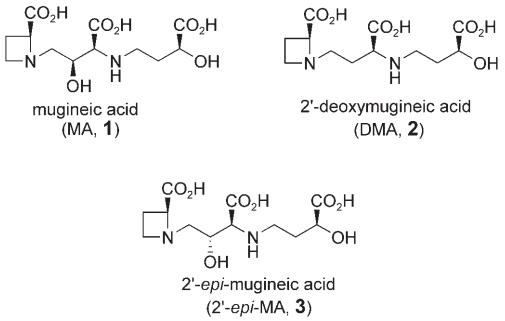
Dedicated to Professor Yoshito Kishi on the occasion of his 70th birthday

Crops grown on alkaline soils, which cover about one-third of the world's land, are prone to iron-deficiency stress because of the low solubility of iron there. To acquire the insoluble iron efficiently, some graminaceous plants have developed a unique strategy characterized by the synthesis and secretion of an iron-chelator phytosiderophore and by a system for the specific uptake of iron(III) in its complex.[1] Mugineic acid (MA, **1**) was first identified as a phytosiderophore in barley[2] and its analogues have since been isolated and identified from various graminaceous species and cultivars;[3] they all form water-soluble 1:1 complexes with iron(III). Owing to its significant implication in plant physiology, this iron-uptake system has been the subject of intensive research since its discovery 30 years ago.[4] However, the mechanistic details of the iron acquisition have not yet been fully elucidated. As a part of our ongoing research program to clarify this mechanism at the molecular level, we have recently identified and characterized the MA·iron(III) transporter HvYS1 from barley, which is the species most tolerant to iron deficiency among graminaceous plants, and we have elucidated the specific localization and function of the encoded protein in the plant.[5] Large-scale expression of the transporter itself and a more detailed mechanistic study thus became our next targets.

The limited supply of phytosiderophores has been a severe bottleneck, particularly for the study of the transport mechanism. To drive the research forward, it is therefore essential to establish an ample supply source of MA (**1**) and/or 2′-deoxymugineic acid (DMA, **2**), the latter of which is a phytosiderophore for rice, wheat, and maize[6] with a similar function of transporting iron(III). In addition, a supply of MA and/or DMA will be potentially important in order to solve the worldwide problem of shortage of food supplies because the use of these compounds may make cultivation possible even on alkaline soils that are not favorable for farming.[7] Herein, we report an efficient, short synthesis of DMA (**2**), which enables the use of this compound in large quantity for biological studies. The potency of DMA in assisting specific iron(III) transport through the transporter HvYS1 was quantitatively estimated. A comparison of the potency of synthetic DMA (**2**) with those of MA (**1**) and its diastereomer, that is, 2′-*epi*-mugineic acid (2′-*epi*-MA, **3**), which were similarly synthesized in this work, clearly proved that these three compounds exhibit the same level of iron-transport ability. New access is, hence, open for the elucidation of the mechanism of phytosiderophore-mediated iron acquisition by plants by using synthetic **2** and its derivatives as probes. The potential value of DMA as a fertilizer or iron supplement can also now be investigated.



The molecular structure of DMA consists of three parts corresponding to α-hydroxybutyric acid, α-aminobutyric acid, and azetidine-2-carboxylic acid. For the preparation of a large quantity of DMA, we chose reductive amination as a key reaction in the assembly of the three amino acid derivatives, because a judicious choice of building blocks and reaction conditions was expected to enable sequential transformations with minimal requirements for workup and purification procedures. Several syntheses of MA and DMA have been reported based on the same principle of reductive amination.[8] There were, however, two major setbacks in the previous syntheses: the extensive use of protecting groups and the cumbersome purification at each step. Another route was also used to assemble the three building blocks, by peptide coupling followed by reduction of the peptide bonds,[9] where the efficiency of synthesis was not satisfactory either. We

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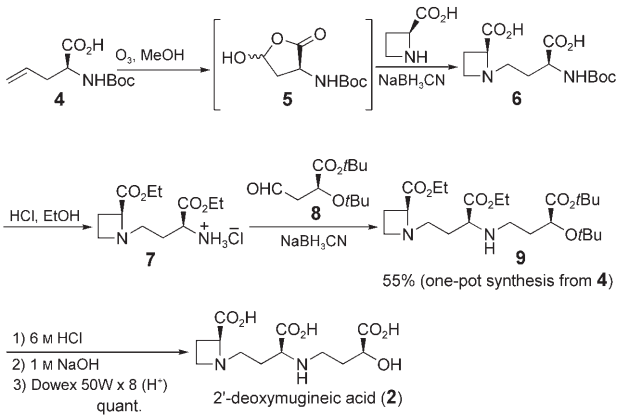
therefore aimed to develop a truly practical synthesis of DMA with minimal use of protecting groups.

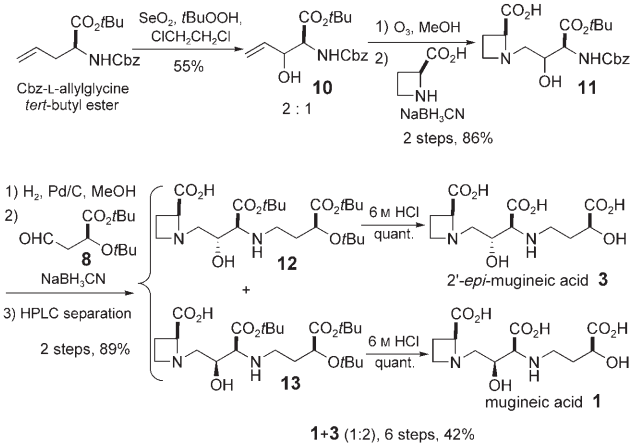
We began the synthesis with commercially available Boc-L-allylglycine (**4**; Scheme 1). After ozonolysis of **4** in methanol at −78 °C, the mixture containing **5** was directly treated with $NaBH_3CN$ and L-azetidine-2-carboxylic acid and warmed to room temperature to give **6**. After evaporation of the reaction solvent (methanol), the residual **6** was treated with dry hydrogen chloride in ethanol. Clean cleavage of the Boc group proceeded in the presence of the remaining excess $NaBH_3CN$ and the two carboxylic acid groups were concurrently converted into ethyl esters to give **7**. The reaction mixture was evaporated to remove excess hydrogen chloride and the residue was treated with a solution of aldehyde **8**[10] and $NaBH_3CN$ in methanol. The second reductive amination also proceeded smoothly to give protected DMA **9**. By extraction of **9** with an organic solvent at this stage, all of the water-soluble byproducts, including amino acids and the excess reducing agent and its degradation products, can be readily removed. Subsequent normal flash chromatography on silica gel afforded the product in a pure state. The protected DMA was obtained in a good overall yield from commercially available **4** through a one-pot synthesis and with just a single chromatography process at the final step. The isolation/purification of any intermediates was not required before that. Finally, the deprotection of **9** gave DMA in quantitative yield. After deionization by ion-exchange resin, recrystallization from EtOH/MeOH/H_2O gave free DMA (**2**).



Scheme 1. One-pot synthesis of a protected 2′-deoxymugineic acid (**9**) from Boc-L-allylglycine (**4**) and deprotection to give free 2′-deoxymugineic acid (DMA, **2**). Boc = *tert*-butoxycarbonyl.

Success in the above synthesis of DMA prompted us to apply the same strategy to the synthesis of MA (**1**), the supply of which has also been greatly required for functional studies. The same starting material, Boc-L-allylglycine, was expected to give **1** after conversion into 2-hydroxy-Boc-L-allylglycine through allylic oxidation and subsequent application of the same series of reactions.

Allylic oxidation of Boc-L-allylglycine ethyl ester by selenium oxide proceeded smoothly. However, this oxidation resulted in the formation of a 9:1 diastereomeric mixture in favor of the unnatural α configuration of the hydroxy group. To obtain an acceptable diastereoselectivity, the allylic oxidation was investigated with several other allylglycine derivatives. Among them, oxidation of Cbz-L-allylglycine *tert*-butyl ester by selenium oxide resulted in the formation of an approximately 2:1 mixture of the 2-hydroxy allylglycine **10** in a modest yield (Scheme 2). Even though the diastereoselectivity at the 2-position was still in favor of the unnatural configuration, the mixed compound **10** was subjected to the subsequent reactions toward the mugineic acid derivatives, as shown in Scheme 2. Ozonolysis of **10** followed by reductive amination with free L-azetidine-2-carboxylic acid gave the desired compound **11** in a good yield as a mixture of diastereomers. Hydrogenolytic deprotection of **11** and subsequent direct reductive amination with aldehyde **8** after removal of the palladium catalyst resulted in a mixture of **12** and **13** in high yield. The diastereomers **12** and **13** were then separated by HPLC (CAPCELLPAK C18-UG80; 35% CH_3CN in H_2O containing 1% AcOH). The isolated diastereomers were separately treated with 6 M hydrochloric acid to give 2′-*epi*-MA (**3**) and MA (**1**), respectively, in quantitative yields. Separation of the deprotected 2′-epimers, **1** and **3**, was difficult even by HPLC under various conditions, so the separation had to be carried out before the final removal of the *tert*-butyl esters.



Scheme 2. Synthesis of mugineic acid (MA, **1**) and its 2′-epimer (2′-*epi*-MA, **3**). Cbz = benzyloxycarbonyl.

With the sufficient quantities of MA (**1**), DMA (**2**), and 2′-*epi*-MA (**3**) in hand, their capacities for iron(III)-complex transport through the HvYS1 transporter expressed in *Xenopus laevis* oocytes[5] were examined. We have reported some results of structure–activity studies for this family of phytosiderophores.[11] Yet the effect of the hydroxy group at the C2′ position was not known. Thus, we evaluated the role of this particular hydroxy group in iron acquisition by measuring electrophysiological transport activity in *Xenopus laevis* oocytes. HvYS1 is a specific phytosiderophore–iron(III) transporter identified from iron-deficient barley roots and belongs to the oligopeptide transporter (OPT) family.[12] The open reading frame (ORF) region of *HvYS1* cDNA was amplified by PCR. In the oocytes injected with cRNA

encoding the ORF region, currents were induced by iron(III) complexes of synthetic compounds **1**–**3** at a level similar to that with natural MA–iron(III) (Figure 1). Currents were absent in water- or noninjected control oocytes.

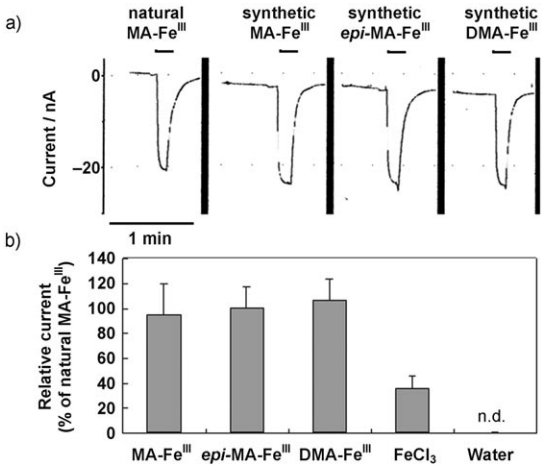


Figure 1. Iron-transporting activities of natural MA, synthetic MA, 2′-*epi*-MA, and DMA. The activity was assessed by two-electrode voltage-clamp analysis with *Xenopus laevs* oocytes. a) Electrogenic iron(III)-complex transport by HvYS1. Each compound was added as the iron(III) complex at a concentration of 50 μM (indicated by the black bars). b) The currents relative to that with natural MA–iron(III) are shown. The error bars show the standard deviation (number of experiments: 5–8). n.d.: not detected.

These results clearly show that iron complexes of these synthetic compounds possess essentially the same transport activity as that of natural MA. The 2′-hydroxy group of MA is hardly responsible for the iron-chelating or transport activity. We also obtained similar results with oocytes injected with cRNA encoding ZmYS1, which was found in maize, was reported as the first example of an iron(III)–phytosiderophore transporter,[13] and is known to transport various phytosiderophore-bound metals including Zn^{2+}, Cu^{2+}, and Ni^{2+} (see the Supporting Information).[14]

The above result that the presence or absence of the 2′-hydroxy group has no definite effect on the iron-transport ability of these phytosiderophores means that DMA, which is now available in sufficient amounts, can be used for the study of the physiological role and mechanism of iron transport by particular transporter proteins. Iron acquisition in the presence of synthetic DMA by a recombinant plant expressing the transporters is already in progress in our laboratory and the results will be soon published elsewhere. Another point to be mentioned is that the 2′-hydroxy group can serve as a potential position for the labeling of mugineic acid analogues for functional studies. The introduction of functionality for affinity labeling on the MA skeleton has, so far, never been successful. The main reason was that all of the previously prepared labeled products lost the ability to form iron(III) complexes owing to the structural modification. We are attempting to avoid this problem by introducing a label at the 2′-hydroxy group of mugineic or 2′-*epi*-mugineic acid.

Experimental Section

9: Ozone was bubbled through a solution of Boc-L-allylglycine (1.2 g, 5.7 mmol) in methanol (40 mL) at −78 °C until the color of the solution changed to blue. After nitrogen was bubbled through until the blue color had gone, L-azetidine-2-carboxylic acid (564 mg, 5.7 mmol) and $NaBH_3CN$ (351 mg, 5.7 mmol) were added to the solution. The mixture was stirred for 2 h at room temperature and then concentrated under reduced pressure. A suspension of the residue in cooled anhydrous HCl/EtOH (prepared from acetyl chloride (4.5 mL) and ethanol (100 mL)) was stirred for 2 h at 0 °C, stirred for 15 h at room temperature, concentrated under reduced pressure, dehydrated by toluene azeotropy, and dried under vacuum for several hours. Aldehyde **8** (1.3 g, 5.7 mmol) and $NaBH_3CN$ (351 mg, 5.7 mmol) were added to a mixture of the residue in methanol (50 mL) at room temperature. The mixture was stirred for 4 h, quenched with sat. $NaHCO_3$, and extracted with ethyl acetate (3 × 200 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (elution with hexane/ethyl acetate (2:1)→ethyl acetate including 0.1% triethylamine) to give protected DMA **9** (1.44 g, 55%) as colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.22$–4.12 (m, 4H), 3.91 (t, $J = 6.4$ Hz, 1H), 3.58 (t, $J = 8.8$ Hz, 1H), 3.39 (br t, $J = 6.0$ Hz, 1H), 3.25 (br t, $J = 6.0$ Hz, 1H), 2.82 (q, $J = 7.6$ Hz, 1H), 2.75–2.61 (m, 2H), 2.59–2.55 (m, 2H), 2.32 (m, 1H), 2.21 (m, 1H), 1.80–1.58 (m, 4H), 1.45 (s, 9H), 1.27 (t, $J = 7.2$ Hz, 3H), 1.26 (t, $J = 7.2$ Hz, 3H), 1.16 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 174.38$, 173.36, 172.07, 80.07, 74.11, 69.68, 64.77, 60.16, 60.11, 59.21, 55.06, 50.68, 43.71, 33.93, 30.65, 27.56, 27.49, 20.96, 13.95, 13.86 ppm; HRMS: *m*/*z* calcd for $C_{24}H_{44}N_2O_7^+$ $[M+H]^+$: 473.3229; found: 473.3221; $[\alpha]_D^{20} = -92.8$ ($c = 1.0$, $CHCl_3$).

2: A suspension of **9** (600 mg, 1.2 mmol) in cooled 6 M HCl (20 mL) was stirred for 10 h at room temperature and concentrated under reduced pressure. A mixure of the residue in 1 M NaOH (30 mL) was stirred for 10 h at room temperature. The mixture was neutralized by 1 M HCl and concentrated under reduced pressure. The residue was purified by column chromatography on an ion-exchange resin (Dowex 50W × 8) (elution with water→1 M NH_3) to give the DMA ammonium salt (360 mg, 99%) as a white solid. Further purification by recrystallization (from H_2O/MeOH/EtOH) gave pure **2** (260 mg, 71%) as a white solid: ^{1}H NMR (300 MHz, D_2O): $\delta = 4.63$ (t, $J = 9.6$ Hz, 1H), 4.01 (dd, $J = 6.6$, 4.5 Hz, 1H), 3.97 (td, $J = 9.6$, 4.2 Hz, 1H), 3.83 (app. q, $J = 9.6$ Hz, 1H), 3.64 (dd, $J = 7.8$, 3.6 Hz, 1H), 3.38–3.18 (m, 2H), 3.14–2.96 (m, 2H), 2.60 (qd, $J = 9.6$, 4.2 Hz, 1H), 2.41 (dt, $J = 11.7$, 9.3 Hz, 1H), 2.18–1.70 ppm (m, 4H); ^{13}C NMR (75 MHz, D_2O): $\delta = 180.05$, 173.21, 172.17, 70.32, 66.90, 59.61, 51.24, 50.50, 44.25, 30.45, 24.54, 21.12 ppm; HRMS: *m*/*z* calcd for $C_{12}H_{19}N_2O_7^-$ $[M-H]^-$: 303.1200; found: 303.1198; $[\alpha]_D^{22} = -69.2$ ($c = 1.0$, H_2O, pH 6.5; literature value: −70.5[6]).

1: ^{1}H NMR (400 MHz, D_2O, pH 4.53 adjusted by the addition of 1 M DCl): $\delta = 4.88$ (t, $J = 9.5$ Hz, 1H), 4.44 (dt, $J = 9.3$, 2.9 Hz, 1H), 4.17 (dd, $J = 4.6$, 7.3 Hz, 1H), 4.10 (dt, $J = 4.2$, 10.1 Hz, 1H), 4.03 (app. q, $J = 9.7$ Hz, 1H), 3.85 (d, $J = 2.9$ Hz, 1H), 3.56 (dd, $J = 9.5$, 13.7 Hz, 1H), 3.43 (dd, $J = 2.7$, 13.7 Hz, 1H), 3.33–3.25 (m, 1H), 3.25–3.16 (m, 1H), 2.77–2.66 (m, 1H), 2.62–2.52 (m, 1H), 2.22–2.14 (m, 1H), 2.09–1.99 ppm (m, 1H); ^{13}C NMR (100 MHz, D_2O, pH 4.53 adjusted by the addition of 1 M DCl): $\delta = 182.3$, 175.8, 171.8, 73.2, 70.6, 67.4 (2C), 59.0, 53.9, 47.9, 32.9, 24.6 ppm; HRMS: *m*/*z* calcd for $C_{12}H_{21}N_2O_8^+$ $[M+H]^+$: 321.1298; found: 321.1292; $[\alpha]_D^{24} = -63.5$ ($c = 0.31$, H_2O; literature values: −70.7,[2] −64.6[8h]).

3: ^{1}H NMR (400 MHz, D_2O, pH 4.53 adjusted by the addition of 1 M DCl): $\delta = 4.89$ (t, $J = 9.5$ Hz, 1H), 4.22 (ddd, $J = 2.7$, 8.3, 9.8 Hz, 1H), 4.16 (dd, $J = 4.4$, 7.6 Hz, 1H), 4.12–4.07 (m, 1H), 4.06 (app. q, $J = 9.7$ Hz, 1H), 3.63 (d, $J = 8.3$ Hz, 1H), 3.57 (dd, $J = 2.7$, 13.2 Hz, 1H), 3.41 (dd, $J = 9.8$, 13.2 Hz, 1H), 3.30 (dt, $J = 12.7$, 7.1 Hz, 1H), 3.16 (dt, $J = 12.7$, 7.1 Hz, 1H), 2.75–2.65 (m, 1H), 2.61–2.51 (m, 1H), 2.20–2.10 (m, 1H), 2.05–1.96 ppm (m, 1H); ^{13}C NMR (100 MHz,

D_2O, pH 4.50 adjusted by the addition of 1M DCl): $\delta = 182.1$, 175.7, 172.4, 73.2, 69.9, 67.5, 67.4, 60.0, 54.4, 48.1, 32.7, 24.5 ppm; HRMS: *m/z* calcd for $C_{12}H_{21}N_2O_8^+$ $[M+H]^+$: 321.1298; found: 321.1298; $[\alpha]_D^{24} = -48.8$ ($c = 0.57$, H_2O).

Electrophysiological studies for *Xenopus laevis* oocytes with synthetic mugineic acid derivative–iron(III) complexes: Two-electrode voltage-clamp analysis of the HvYS1 transporter in *Xenopus laevis* oocytes was performed as described previously.[5] The ORF region of *HvYS1* cDNA (DNA data base of Japan (DDBJ) under the accession number AB214183) was amplified by PCR with the forward primer 5′-GCTCTAGACCACCATGGACATCG-3′ and the reverse primer 5′-CGCGGGATCCTTAGGCAGCAGGTAG-3′; it was then inserted into the *Xba*I,*BamH*I site of *Xenopus* expression vector pSP64 polyA. The plasmid was linearized with *BamH*1 and then the cRNA was transcribed in vitro with SP6 RNA polymerase. The cRNA solution (50 nL, 0.05 $\mu g\,\mu L^{-1}$) was injected into *Xenopus* oocytes, which then were incubated for 2–4 days at 16 °C in ND96 buffer (pH 7.6) containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES). The oocytes were voltage clamped at −60 mV (Oocite clamp OC-725c, Warner Instruments) and steady-state currents were obtained in response to the addition of the MA–, 2′-*epi*-MA–, or DMA–iron(III) complexes (10 μL, 7.5 mM; final concentration: 50 μM). The transport activity was analyzed with Origin 6.1 software (Microcal Software).

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