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CO-OCCURRENCE OF NICOTIANAMINE AND AVENIC ACIDS IN *AVENA SATIVA* AND *ORYZA SATIVA*

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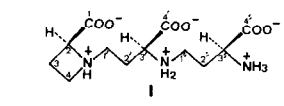
Key Word Index—*Avena sativa*; *Oryza sativa*; Gramineae; iron deficiency; iron transport; iron chlorosis; iron chelating amino acids; nicotianamine.

Abstract—An amino acid derivative isolated from seedlings of *Avena sativa* and *Oryza sativa*, along with avenic acid A and its derivatives which possess a chelating ability with iron ions, has been shown to be nicotianamine. The co-occurrence of nicotianamine and avenic acids in the same plant, as well as their structural similarity, reveals their close biosynthetic relationship.

INTRODUCTION

Takagi first reported [1] the detection of amphoteric iron chelating substances which might play a role in iron uptake and transport in the root washings of rice and oat plants cultured under iron deficient conditions. Such substances with a chelating activity, mugineic acid (**2**) [2, 3], avenic acid A (**3**) [4], 3-hydroxymugineic acid (**4**) [3] and 2′-deoxymugineic acid (**5**) [3] have so far been isolated from the root washings of *Hordeum vulgare*, *Avena sativa and Secale cereale*. On the other hand, nicotianamine (**1**) which possesses a chemical structure closely related to that of mugineic acid (**2**) and its relatives has been isolated from a variety of plant sources such as *Nicotiana tabacum* and *Fagus sylvatica* [5–7]. Very recently, Buděšínský *et al.* [8] have also reported the isolation of this compound from *Beta vulgaris* and *Medicago sativa* as the normalizing factor in the mutant 'chloronerva' of *Lycopersicon esculentum* cv Bonner Beste and they demonstrated the general occurrence of this compound in vascular plants.

During the course of our investigation to isolate new compounds related to the iron chelating amino acid derivatives, we isolated an amino acid derivative from the ethanol extract of seedlings of *A. sativa* and *Oryza sativa* cultured under iron sufficient conditions and the compound has been shown to be nicotianamine (**1**). Here we describe the isolation and characterization of this substance.



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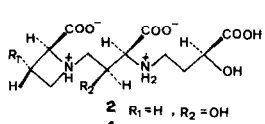
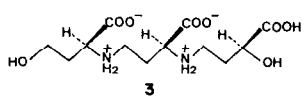


2 R_1=H, R_2=OH
4 R_1=OH, R_2=OH
5 R_1=H, R_2=H



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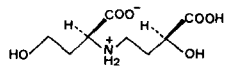

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RESULTS AND DISCUSSION

In the root washings of *A. sativa* cultured under iron deficient conditions, the iron chelating amino acids, avenic acid A (**3**) and 2′-deoxymugineic acid (**5**) were specifically observed and no other amino acid was detected. These iron chelating amino acids showed a positive reaction to the *o*-phenanthroline method [1]. On the other hand, a minor active amino acid, avenic acid B (**6**) [9], has been isolated in addition to the above mentioned two iron chelating amino acids from the ethanol extract of *A. sativa* cultured under the same conditions, and many other amino acids have been detected by means of PC and HPLC analysis in the extract. In the ethanol extract of the leaves, an unusual amino acid derivative which showed a negative reaction to *o*-phenanthroline and a brown to violet coloration with ninhydrin was observed. This non-protein amino acid (R_f, 0.03 on PC; R_t, 15 min on HPLC) was found to be a neutral amino acid from the behavior patterns on ion exchange resins and was also detected more abundantly in the ethanol extract of seedlings of *A. sativa*. The isolation and purification of this amino acid was achieved according to the following procedures from the ethanol extract of *A. sativa* cultured until full expansion of the first leaf under iron sufficient conditions.

The neutral amino acid fraction obtained from the extract in the usual manner was subjected to gel filtration using Sephadex G-10, monitoring the separation by HPLC. The fractions containing the amino acid were separated on a Dowex 50W×4 column which was buffered with an ammonia–formate buffer (pH 3.05). Desalting the fractions, which showed a single peak on HPLC, gave the non-protein amino acid derivative (9.8 mg) as a white powder. This amino acid decomposed above 253°, $[\alpha]_D$ −45.0° (H_2O; *c* 0.13) and has the molecular formula $C_{12}H_{21}N_3O_6$, mass spectrum (FD); *m/z* 320 $[M+NH_3]^+$. The IR and NMR spectra of the amino acid were found to be identical to those of nicotianamine (**1**) [5, 6]. For the purpose of direct comparison, an

authentic sample of **1** was isolated from young leaves of *Lycium chinence* where it is found in high concentrations [7]. Consequently, the unusual *Avena* amino acid derivative was shown to be identical with **1** in all respects including PC R_f value, R_t on HPLC, mp, $[\alpha]_D$, IR and ^1H NMR spectra. Nicotianamine (**1**) was also isolated from *O. sativa* by the same method. Recently, we have synthesized optically pure **1** from L-azetidine-2-carboxylic acid and L-aspartic-β-semialdehyde derivatives [10].

As mentioned above, the iron chelating amino acids, avenic acid A (**3**) and 2'-deoxymugineic acid (**5**) were isolated from the root washing and root extract of *A. sativa* grown in an iron free medium but these amino acids were not detected in the seedling plant grown under normal conditions. On the other hand, **1** was isolated more abundantly from *A. sativa* cultured under normal conditions than from those grown in iron-free conditions. Although a biosynthetic relationship between the iron chelating amino acids and **1** is not certain, these findings together with similarity of their structure suggest a close relationship in their biosynthetic pathway. One possibility is that the iron chelating amino acid [e.g. 2'-deoxymugineic acid (**5**)] is biosynthesized by a transamination reaction and a reduction of the terminal amino group of **1**. Another is that a hydroxy acid such as malic semialdehyde is used as a building block in the biosynthesis instead of aspartic semialdehyde. This hypothesis is now being tested.

The biological significance of **1** is interesting because of its wide distribution. Recently, Buděšínský *et al.* reported [8] that it served as a normalizing factor in the tomato mutant 'chloronerva' and was presumed to be an iron chelator. They suggest that **1** is a possible phytosiderophore of general importance in cellular iron transport and/or metabolism. Mugineic acid (**2**), the representative iron chelating amino acid, proved to be inactive as a normalizing factor [Scholz, G., personal communication]. The difference between the biological activity of the iron chelating amino acids and **1** is very interesting and studies concerning the biosynthetic pathway and iron chelating activity of **1**, mugineic and avenic acids are proceeding.

EXPERIMENTAL

All mps were uncorr. IR spectra refer to KBr disks. ^1H NMR spectra were recorded as D_2O solns at 100 MHz with TSP as int. standard. PC was performed using *n*-BuOH–HOAc–H_2O (4 : 1 : 5 upper layer) by the ascending technique on Toyo No. 50 paper. HPLC was carried out on an instrument equipped with a refractive index detector. A stainless steel column packed with Hitachi gel 2618 (cation exchange resin) was used and eluted with NH_3–HCO_2H buffer (pH 3.05) at a rate of 0.5 ml/min.

Isolation of **1**. Leaves and roots of *A. sativa* L. (5000 plants) cultured until the first leaf fully expanded were homogenized with 70% aq. EtOH. After filtration of the homogenate, the combined filtrates were evaporated *in vacuo*. The residue was extracted with EtOAc and the aq. layer applied to Amberlite IR-120 B resin (H^+, 20–50 mesh, 3.6 × 30 cm). After washing with H_2O, the column was eluted with 1 N pyridine (1 l.), affording the acidic and neutral amino acids. The column was subsequently eluted with 1 N NH_3 (1 l.) to give the basic amino acids. The acidic and neutral amino acid fraction was applied to Dowex 1 × 8 resin (AcO^-, 50–100 mesh, 2.6 × 30 cm). After washing with H_2O (0.8 l.) to give the neutral amino acids, the column was eluted with 1 N HOAc to yield the acidic amino acids. The neutral amino acid fraction was applied to a Sephadex G-10 column (1.6 × 100 cm) and eluted with H_2O (3 ml fractions). Each fraction was monitored by HPLC. The fractions containing **1** were collected and subjected to chromatography on Dowex 50W × 4 resin (200–400 mesh, 0.8 × 45 cm, buffered with a pH 3.05 NH_3–HCO_2H buffer) eluting with the same buffer (8 ml fractions). The fractions which showed a single peak on HPLC were collected and desalted using Dowex 50W × 4 (100–200 mesh) to yield **1** as a white powder (9.8 mg). Mp decomp. above 235°, $[\alpha]_D$ −45.0° (H_2O; *c* 0.13), R_f on PC 0.03, R_t on HPLC 15 min. The isolated **1** was found to be identical in all respects (R_f on PC, R_t on HPLC, mp, $[\alpha]_D$, IR and ^1H NMR to an authentic specimen of nicotianamine.

1 was aslo isolated from the seedlings of *A. sativa* (5000 plants) grown in nutrient soil (Kureha-Baido supplied by Kureha Chemical Industry Co. Ltd) and from seedlings of *O. sativa* (5000 plants) (10.0 mg and 12.8 mg respectively).

Avenic acid A (**3**), 2'-deoxymugineic acid (**5**) and Fe chelating amino acids were not detected in the extract of seedlings by means of the *o*-phenanthroline method by PC and HPLC.

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