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NICOTIANAMINE, A POSSIBLE PHYTOSIDEROPHORE OF GENERAL OCCURRENCE*

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Key Word Index—*Beta vulgaris*; Chenopodiaceae; *Lycopersicon esculentum*; Solanaceae; *Medicago sativa*; Leguminosae; 'normalizing factor' for the tomato mutant '*chloronerva*'; non-protein amino acid; (2*S*:3′*S*:3″*S*)-*N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid; nicotianamine; pytosiderophore; chelating agent for Fe^{3+} and Cu^{2+}; iron transport and metabolism in plants.

Abstract—The 'normalizing factor' for the mutant '*chloronerva*' of the tomato, *Lycopersicon esculentum* cv Bonner Beste was shown to possess the structure (2*S*:3′*S*:3″*S*)-*N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2- carboxylic acid and proved to be identical with nicotianamine, especially on the basis of its high resolution mass and NMR spectroscopic investigations and those of some of its derivatives. It seems to be of general occurrence in vascular plants and has been isolated from *Medicago sativa* (Leguminosae) and *Beta vulgaris* (Chenopodiaceae) using a large-scale isolation procedure. Nicotianamine has an optimal molecular structure for chelating iron ions and is considered a possible phytosiderophore with an essential function in cellular iron transport and/or metabolism.

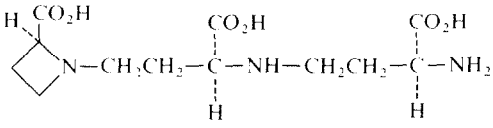
INTRODUCTION

In 1960 a recessive, semi-lethal mutant '*chloronerva*' of *Lycopersicon esculentum* Mill. cv Bonner Beste (Solanaceae) was described [1] exhibiting a severe growth and developmental inhibition, as well as a chlorophyll defect in intercostal areas of young leaves. The phenotype of the original tomato wild type could be completely restored or 'normalized' by grafting mutant scions upon wild type rootstocks or by application of extracts from unmutated plants to the leaves of the mutant [1, 2]. Biochemical experiments revealed a disturbed iron metabolism of the mutant, leading to an excessive iron absorption by the roots on the one hand and an irregular iron distribution within young leaves on the other [3, 4]. Results from grafting experiments and plant extract applications indicated the existence of a water-soluble substance, i.e. the 'normalizing factor' for the tomato mutant '*chloronerva*' which was finally isolated from alfalfa (*Medicago sativa* L., Leguminosae) [5, 6] and later from *Cardaria draba* (L.) Desv. (Cruciferae) [7]. About 1 μg of this 'normalizing factor' per mutant plant yielded a positive response after application to the leaves. The substance gave a positive reaction with ninhydrin; its MW was estimated to be in the range 350–500 according to gel chromatography [6]. Screening experiments suggested that the factor is present in all vascular plants, including fern sporophytes, but absent in non-vascular species, thus indicating possible participation in processes of plant organization and/or of iron transport in higher plants [8]. Complex formation of the 'normalizing factor' with trivalent iron and bivalent copper was demonstrated [9]. The present paper describes the large-scale isolation and identification of this substance.

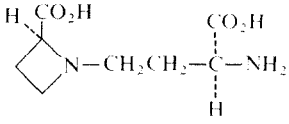
RESULTS AND DISCUSSION

A large-scale procedure for the isolation of the 'normalizing factor' was developed making it possible to obtain larger amounts of pure, crystalline substance from aerial parts of alfalfa (*Medicago sativa* L.) and sugar beet (*Beta vulgaris* L. convar. *crassa* var. *altissima* Döll.). High resolution mass spectrometry of the tetra-(trimethylsilyl) derivative gave the molecular composition $C_{24}H_{53}N_3O_6Si_4$ for $M^{+\cdot}$ (*m/e* 591) and hence $C_{12}H_{21}N_3O_6$ for the 'normalizing factor' [10]. This formula is in accordance with the elemental analysis of a sample dried at 180° *in vacuo* and equivalent weight determination by potentiometric titration. Further important data concerning the 'normalizing factor' were obtained from NMR studies. The ^{13}C NMR spectrum corroborated the assumed number of carbon atoms and also threw light on their character. The ^1H NMR spectra in D_2O made it possible to account for 15 non-exchangeable hydrogen atoms and three isolated CH_2CH_2CH groups, of which one is very probably in a

*Part 10 in the series "The 'Normalizing Factor' for the Tomato Mutant '*chloronerva*'". For Parts 1 to 9, see refs. [1–9].



1 (Nicotianamine)



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four-membered ring. Together with the MS fragmentations of the 'normalizing factor' itself, as well as its tetra-(trimethylsilyl) derivative, its di-(4-bromobenzoyl) trimethyl ester and diacetyl methyl ester derivative, and some degradation experiments, these data indicated that the structure of the 'normalizing factor' was (2*S*:3′*S*:3″*S*)-*N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid [10]. This structure is identical with the structure of the unusual amino acid nicotianamine (**1**) [11, 12], isolated some years ago from tobacco leaves (*Nicotiana tabacum* L.) [11] and beechnuts (*Fagus silvatica* L.) [12] and detected in other plants [13] as well. Comparison of the physical constants, IR spectrum, TLC behaviour and biological activity of the 'normalizing factor' with the data of authentic nicotianamine, as well as a comparison of the ^{13}C and ^{1}H NMR spectra of our material with the literature values for nicotianamine [11, 12] proved the identity of the 'normalizing factor' to be **1**.

As is evident from a Dreiding model, nicotianamine (**1**) has an optimal molecular structure for complex formation with iron (Fig. 1). Not only are six functional groups present, necessary for octahedral coordination, but the distances between the groups are also optimal for the formation of chelate rings: Three 5-membered rings formed by the α-amino acid residues and two 6-membered rings formed by the 1,3-diaminopropane moieties. The special location of the oxygen atoms on one side of the complex and the methylene groups and/or the azetidine ring system on the other might play a decisive role in the biological function of the complex. The complex formation between **1** and Fe^{3+} ions has been convincingly proved on the basis of the appearance of a positive Cotton effect at *ca* 250 nm (pH 4.5, in H_2O) [9]. An analogous Cotton effect at 265 nm (pH 9, in H_2O; UV_{max} at 261 nm) was also observed with Cu^{2+} ions, having, in contrast to other *S*-amino acids [14], a positive sign indicating special structural relations. When the concentration of copper ions was kept constant and that of nicotianamine increased, the size of the Cotton effect increased until a proportion of one molecule of nicotianamine per Cu^{2+} ion was reached. That means that a 1:1 complex is formed. The ORD curve in the visible region shows a peak at 560 nm (UV_{max} at 650 nm). Like other *S*-amino acids [15], nicotianamine itself shows a positive Cotton effect (peak at 223 nm, pH 4, in H_2O).

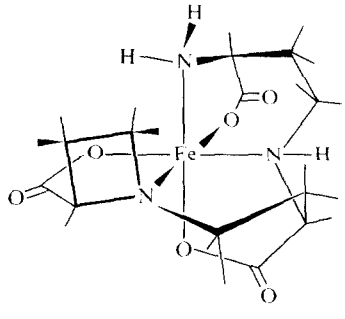

Fig. 1. Dreiding model of the iron nicotianamine complex.

While nicotianamine (**1**) was active in the biological test after application of *ca* 1 μg per plant, *S*-azetidine-2-carboxylic acid or (2*S*:3′*S*)-*N*-(3-amino-3-carboxypropyl)-azetidine-2-carboxylic acid (**2**) [12] proved to be inactive.

According to our present knowledge [1–13], nicotianamine (**1**) is an essential constituent of higher plants and is considered to be a possible specific phytosiderophore of general importance for the cellular iron transport and/or metabolism ([16–18] cf. [19–21]).

EXPERIMENTAL

Nicotianamine (**1**). For the isolation on a pilot plant scale the modified procedure [7] of the original isolation scheme [6] was used. Further minor changes were introduced, and the final procedure consisted of the following steps: (a) exposure of the freshly harvested plant material to $CHCl_3$ for *ca* 20 min; (b) expression of the juice in a press (yield of juice *ca* 50–60% of the fresh plant weight); (c) precipitation of proteins by heating at 80° for 1 hr and filtration through Celite on a centrifuge; (d) decolorization by filtration through a bed of Wofatit EZ resin (weakly basic anion exchanger); (e) retention of substances containing amino groups on a strongly acid sulfonated cation exchanger resin in H^+-form, washing with distilled H_2O and elution with 2 N NH_3; (f) concn of the eluate *in vacuo*; (g) purification of the concentrate (in portions) by Sephadex G-25 chromatography with 0.05 N NH_3; **1** moved fast, but the zone was not sharp and the later fractions had to be rechromatographed; (h) concn *in vacuo* of the combined enriched fractions from the preceding operation; (i) chromatography on Si gel (Si gel according to refs. [22, 23], particle size 120–200 μm), packed in EtOH, using a linear H_2O-in-EtOH gradient (starting with 70% EtOH, finishing with 30% EtOH); (j) elimination of silicic acid from fractions containing **1** by filtration through a small column of cation exchanger (see above), washing with distilled H_2O and elution with 2 N NH_3; (k) concn of the eluate and precipitation with a 6-fold amount of EtOH; (l) crystallization from H_2O containing 0.005% Thiomersal (VEB Berlin-Chemie). Chromatographic fractions were analysed either by 2D-PC [5] or, in the case of purer fractions, by TLC on Si gel with *n*-PrOH–H_2O (6:7), detection with ninhydrin. **1** was isolated from aerial parts of alfalfa and leaves of sugar beet in yields of 0.0002–0.001% and *ca* 0.00025%, respectively. The samples obtained proved to be identical in every respect with authentic nicotianamine. Crystals with decomp. above 250° and $[\alpha]_D^{20}$ −49.7° (H_2O, *c* 1.09), lit.: −60.5° (H_2O) [11], −50° (H_2O) [12]. (After drying at 180° *in vacuo* found: C, 47.1; H, 6.9; N, 13.8. Calc. for $C_{12}H_{21}N_3O_6$: C, 47.5, H, 7.0, N, 13.9%). MS (EI) 70 eV *m/e* (rel. int.): 285 (0.2), 267 (1), 250 (13), 197 (3), 192 (9), 169 (13), 141 (11), 123 (9), 114 (21), 101 (8), 99 (70), 84 (22), 71 (53), 56 (100). MS (CI, NH_3) *m/e* (rel. int.): 268 (53), 250 (30), 185 (58), 101 (18), 99 (98), 71 (52), 56 (100). MS (CI, *i*-C_4H_{10}) *m/e* (rel. int.): 268 (47), 185 (100), 101 (32).

Equivalent weight determination. **1** (29.020 mg) containing 7.29% H_2O was dissolved in 10 ml H_2O, and 5 ml 8% aq.

formaldehyde were added. Automatic titration was carried out with a titrator TTT Ic (Radiometer, Copenhagen), using 0.1 N NaOH ($f = 1.0153$). The recorded potentiometric curve had only one well discernible inflexion point at 0.863 ml consumption. The second, hardly detectable inflexion point was at double consumption. From these data the MW 307 was calculated. When **1** was titrated with 0.05 N $HClO_4$ in HOAc, under the assumption that two basic groups were titrated, approximately the same MW (310) was obtained.

Biological test. Young seedlings of *Lycopersicon esculentum* cv Bonner Beste, mutant '*chloronerva*' with their first leaves expanding were selected for uniformity and set up in a greenhouse. Every soln to be tested was applied 3 times per day with a smooth brush to the chlorotic leaflets of five seedlings. The response was considered positive when a change of the chlorotic leaflets to a normal green colour was observed, which happened in most cases within 1 week. A water control was included for comparison. All substances were dissolved in 0.02% Tween 20 as wetting agent. They were stored at 2° and transferred to the greenhouse only 30 min before application.

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