Pure & Appl. Chem., Vol. 58, No. 5, pp. 745–752, 1986.
Printed in Great Britain.

Identification and characterization of an endogenous cytometallophore of general distribution in plants

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Abstract - Normal growth and development of higher plants is dependent on the presence of small amounts of a compound which seems to be of general distribution in the plant kingdom, at least in vascular plants. This compound was shown to possess the structure (2S:3'S:3"S)-N-/ N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl/-azetidine-2-carboxylic acid (1) and proved to be identical with nicotianamine. The only plant until yet known showing a block in the biosynthesis of nicotianamine is the monogenic, semi-lethal mutant 'chloronerva' of the tomato Lycopersicon esculentum Mill. which is completely normalized restoring the phenotype of the original tomato wild-type by exogenous application of 1 demonstrating the general significance of this unique non-proteinous amino acid. Nicotianamine possesses an optimal molecular structure for chelating iron(II) and other bivalent transition-metal ions (e. g. Cu, Ni, Co, Zn, and Mn). The optical antipode of 1, the (+)-nicotianamine as well as a number of structurally related compounds have been synthesized and their activity as "normalizing factor" for the mutant 'chloronerva' investigated. According to our present knowledge, nicotianamine is considered to be a specific cytometallophore with an essential function in the cellular transport and metabolism of bivalent transition-metal ions in plants, however not only as carrier but also as regulator of these and other processes.

INTRODUCTION

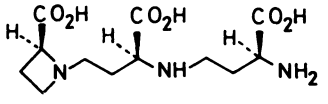
In 1960 a spontaneous recessive, monogenic mutant 'chloronerva' of Lycopersicon esculentum Mill. cv Bonner Beste (Solanaceae) was described (ref. 1) exhibiting a severe growth and developmental inhibition, as well as a chlorophyll defect in the intercostal areas of young leaves. Flower buds are very rarely developed but don't unfold and die off. Normal growth and development, that means the phenotype of the original tomato wild-type could be, however, completely restored by grafting upon normal rootstocks or by application of extracts from unmutated plants to the leaves of the mutant (ref. 1 and 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 the young leaves on the other hand (ref. 3 and 4). These results indicated the lack of an essential constituent in the mutant as well as the existence of a water-soluble substance with phenotypically normalizing properties in non-mutated plants.

With the aim to isolate this "normalizing factor" for the tomato mutant 'chloronerva', to elucidate its chemical structure, and to clear up its physiological function for the plant organism a co-operative programme has been started joining laboratories of the Central Institute of Genetics and Cultivated Plant Research at Gatersleben and the Institute of Plant Biochemistry at Halle of the Academy of Sciences of the German Democratic Republic, of the Institute of Organic Chemistry and Biochemistry at Prague of the Czechoslovak Academy of Sciences as well as of the Institute of Organic Chemistry of the University of Cologne of the Federal Republic of Germany (for some review articles see ref. 5 - 7).

ISOLATION AND STRUCTURAL ELUCIDIATION OF THE "NORMALIZING FACTOR" AND ITS IDENTIFICATION WITH NICOTIANAMINE

On the basis of some preliminary investigations (ref. 8), the "normalizing factor" for the mutant 'chloronerva' was finally isolated in a crystalline state from alfalfa (Medicago sativa L., Fabiaceae) (ref.9 and 10), from Cardaria draba (L.) Desv. (Cruciferae), and from the sugar beet (Beta vulgaris L. convar. crassa var. altissima Döll., Chenopodiaceae) (ref. 10). The large-scale isolation procedure consists in a number of extraction and separation steps using Celite, ion-exchange, Sephadex G-25, and silica-gel chromatography (cf. ref. 11).

Only about 1 µg of the pure, crystalline substance per mutant plant yielded a positive response after application to the leaves of the mutant. It gave a positive reaction with ninhydrin, its molecular mass was estimated to be in the range 350 - 500 according to gel chromatography (ref. 9), and a complex formation with iron and copper ions was demonstrated (ref. 12).



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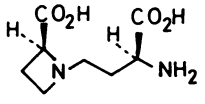

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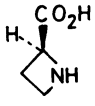

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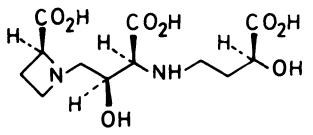

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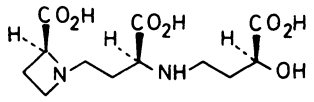

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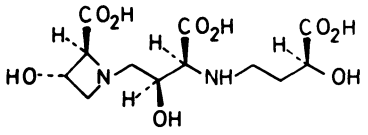

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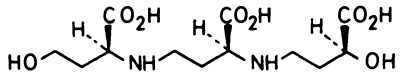

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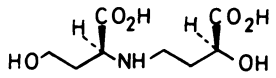

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High-resolution mass spectrometry of its tetra(trimethylsilyl) derivative led to the molecular composition $C_{24}H_{53}N_3O_6Si_4$ for this derivative and hence $C_{12}H_{21}N_3O_6$ for the "normalizing factor" (ref. 13). Further important data concerning its structure were obtained from NMR studies. Thus, the ^{13}C NMR spectrum corroborated the assumed number of carbon atoms and also threw light on their characters. The high-resolution ^1H NMR spectra in D_2O made it possible to account for 15 non-exchangeable hydrogen atoms and three isolated CH_2CH_2CH groups, one of which is very probably in a four-membered nitrogen containing ring. Together with the mass spectrometric fragmentation patterns of the "normalizing factor" itself as well as its tetra(trimethylsilyl) derivative, its bis(4-bromobenzoyl)trimethyl ester and diacetyl-methyl ester derivatives, and some degradation experiments, these data indicated that the structure of the isolated biologically active compound is (2S:3'S:3"S)-N-/N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl/-azetidine-2-carboxylic acid (1) (ref. 10 and 13). This structure is identical with that of the unusual, non-proteinous amino acid nicotianamine, isolated some years ago from tobacco leaves (Nicotiana tabacum L., Solanaceae) (ref. 14) and beechnuts (Fagus silvatica L., Fagaceae) (ref. 15) which was confirmed by comparison with an authentic sample.

DISTRIBUTION OF NICOTIANAMINE IN THE PLANT KINGDOM

According to the results obtained by isolation in a preparative scale (ref. 8 - 10, 14 - 16) or by screening experiments using an automatic amino acid analyzer (ref. 17 - 20) nicotianamine (1) was detected in more than 50 species from 27 plant families especially belonging to the spermatophyta (cf. ref. 5 and 6). However, according to recent investigations (ref. 20), some multi-

cellular sporophyta were shown to contain also nicotianamine, e. g., a higher fungus (Polyporus sp.), some mosses and liverworts as well as a lichen (Parmelia sp.). Up to now, the tomato mutant 'chloronerva' is the only higher plant known where nicotianamine is absent, due to a metabolic block in the biosynthesis of this amino acid (ref. 21).

The distribution of 1 in different organs shows some differences. Generally, its highest content is in young growing tissues and its minimal concentration within the seeds (ref. 17 and 18). The average concentration of nicotianamine in leaves is 0.1 - 2.0 µmol/g dry weight (ref. 5). Also cell cultures of Lycopersicon esculentum are able to synthesize this amino acid (ref. 22).

OCCURRENCE OF AMINO ACIDS WITH STRUCTURES ANALOGOUS TO THAT OF NICOTIANAMINE

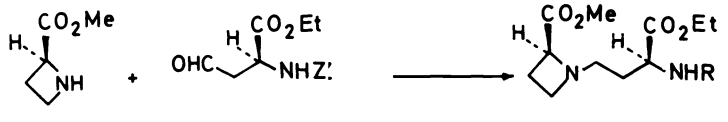
Nicotianamine (1) is a derivative, that means the trimeric form of (S)-azetidine-2-carboxylic acid (3). This amino acid was shown to occur in species of Liliaceae and Agavaceae (ref. 23) as well as in Delonix regia (Boj.) Raf. (ref. 24), Beta vulgaris L. (ref. 25), and Nicotiana tabacum L. (ref. 26), while its dimeric form, the (2S:3'S)-N-(3-amino-3-carboxypropyl)-azetidine-2-carboxylic acid (2), was isolated from seeds of Fagus silvatica L. (ref. 15). The precursor for the biosynthesis of azetidine-2-carboxylic acid (3) and its di- and trimerization products 2 and 1 is considered to be (S)-methionine (ref. 27).

Independent of our investigations, Japanese scientists were able to detect (ref. 28) and to isolate (ref. 29 - 35) several amino acids possessing chelating properties for iron and other metal ions from root washings of gramineous plants grown under iron-deficient conditions. These amino acids were shown to possess structures strongly related to nicotianamine (1), thus mugineic acid (4) from barley (Hordeum vulgare L.) (ref. 29 and 30), 2'-deoxymugineic acid (5) from oat (Avena sativa L.) (ref. 30) and wheat (Triticum aestivum L.) (ref. 31 and 32), 3-hydroxymugineic acid (6) from rye (Secale cereale L.) (ref. 30), as well as avenic acid A (7) and B (8) from oat (ref. 33 - 35). Most probably, the acids 4 - 8, at least in the investigated plants, are responsible for the iron uptake by the roots and seem to be metabolites of nicotianamine (1).

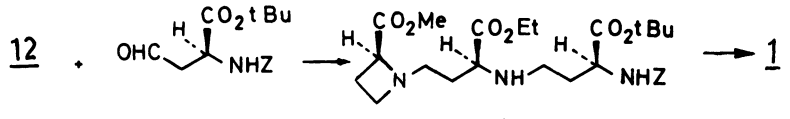
SYNTHESIS OF NICOTIANAMINE AND SOME STRUCTURALLY RELATED COMPOUNDS

Heating of an aqueous solution of (S)-azetidine-2-carboxylic acid (3) with half an equivalent of sodium hydroxide to 100 °C for 24 h gave, in addition to the dimeric acid 2, natural (-)-nicotianamine (1) in 4 % yield (ref. 15). This synthesis established the configurations at all chiral C-atoms of 1 as S. Starting from (R)-azetidine-2-carboxylic acid, the enantiomeric (+)-(R,R,R)-nicotianamine was obtained in an analogous way in 2 % yield together with the optical antipode of the dimeric acid 2 (ref. 36).

A total synthesis of 1 was published by Japanese authors (ref. 33 and 37). (S)-2-Methoxycarbonylazetidine (9) was coupled with protected (S)-aspartic-β-semialdehyde (10) to 11 by means of sodium cyanoborohydride. After elimination of the benzyloxycarbonyl residue, 12 reacted with the aldehyde 13 to give the nicotianamine derivative 14, which - after catalytic hydrogenation, followed by treatment with trifluoroacetic acid and finally with potassium hydroxide - yielded nicotianamine (1).

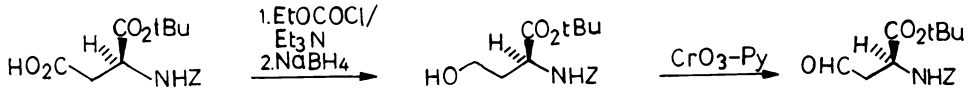


9 10 11 : R = Z 12 : R = H·HCl

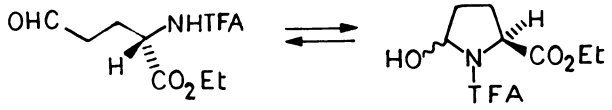


13 14

The published synthesis of the protected (S)-aspartic-ß-semialdehyde 13 (ref. 33, 37, and 38) is not easily accompliched especially in a larger scale. However, we were able to realize a simple synthesis of 13 starting from (S)-aspartic acid (ref. 39, cf. 40). As shown in the formula pictures, the protected aspartic acid 15 has been reduced to the homoserine derivative 16 which was re-oxidized by dipyridine chromium(VI) oxide leading to 13.



15 16 13



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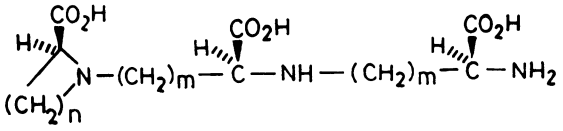
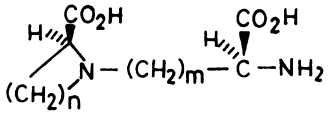
In sequences of reactions analogous to 9 ⟶ 14 ⟶ 1 using the protected (S)-aspartic-semialdehyde 13, the related N-trifluoroacetyl-ethyl ester derivative or its homologue ethyl (S)-5-oxo-2-(trifluoroacetylamino)pentanoate (17) we synthesized the nicotianamine homologues 18 - 20 and the respective dimeric amino acids 21 - 23 (ref. 41 and 42). The corresponding peptide analogues 24 - 29 have also been synthesized from the respective protected amino acids using the EEDQ or the DCC method (ref. 43).

The biological activities of all the synthesized homologues and analogues have been investigated with regard to their ability to normalize the tomato mutant 'chloronerva' (cf. the last chapter).



18-20



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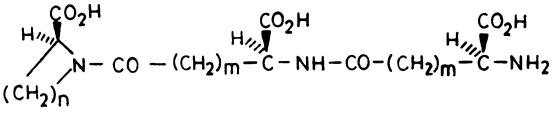

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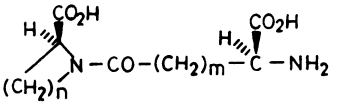

27-29

	n	m		n	m
18, 21	3	2	24, 27	2	1
19, 22	2	3	25, 28	3	1
20, 23	3	3	26, 29	3	2

METAL CHELATING PROPERTIES OF NICOTIANAMINE AND ANALOGOUS AMINO ACIDS

As shown by Dreiding-model considerations, nicotianamine (1) has an optimal molecular structure for complex formation with iron ions (Fig. 1) (ref. 10). Not only are six functional groups present, necessary for a hexadentate 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.

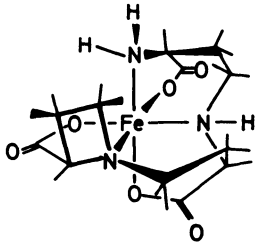

Fig. 1. Structure of the iron(II)-nicotianamine (1) complex.

According to X-ray structural analyses, the copper(II), iron(III), and cobalt(III) complexes of mugineic acid (4) show quite analogous structures (ref. 44 - 48) as assumed for the iron complex of nicotianamine (1) (Fig. 1) (ref. 10), confirming the hexadentate coordination forming a distorted octahedral geometry.

Surprisingly, the chelating properties of the two related amino acids nicotianamine (1) and mugineic acid (4) show remarkable differences due to the presence of the terminal primary amino group in 1 instead of the hydroxy group in 4 (ref. 47). Apart from the comparable stability of their Cu(II) complexes, the stability constants for the Fe(II) and Zn(II) complexes of 1 are much larger than those of the respective complexes of 4 (Table 1). On the other hand, despite of the relatively high stability constant of the Fe(III) complex of 4 (log K = 18.1), a Fe(III) complex of nicotianamine (1) was not observed under the experimental conditions used by ourselves (ref. 47). The resultant values of the stability constants of the metal(II)-nicotianamine complexes (Table 1) follow the Irving-Williams rule of formation constants, i. e. the sequence of stability is $Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)$.

TABLE 1. Stability constants (log K) of the metal-nicotianamine (1) and metal-mugineic acid (4) complexes.

Complex	Cu(II)	Ni(II)	Co(II)	Zn(II)	Fe(II)	Fe(III)	Mn(II)	Mg(II)
of 1 (ref. 47)	18.6	16.1	14.8	14.7	12.1	-	8.8	≈4.5
of 4 (ref. 45)	18.3			10.7	8.1	18.1		

The iron($Fe(OH)_3$)-solubilizing abilities of mugineic acid (4) and 2'-deoxy-mugineic acid (5) as well as the observed iron uptake by roots of rice plants effected by both acids were shown to be much higher in comparison to those figures obtained with nicotianamine (1) which were comparable to the control (ref. 46).

Most microbial siderophores possess hydroxamate or catecholate groups as Fe(III)-ligand donors but generally show larger stability constants as the above-mentioned phytometallophores, e. g., ferrichrome (log K = 29.1), ferrioxamine B (log K = 30.6) (ref. 48), and ferric enterobactin (log K ≈ 52) (ref. 49 and 50). However, mugineic acid (4) and especially nicotianamine (1) are much better complexing agents for ferrous ions than the microbial siderophores of the hydroxamate or catecholate type. Nicotianamine (1) is probably the lowest molecular weight natural product found so far which is capable of forming an intramolecular 1 : 1 hexadentate complex with iron(II) and other bivalent transition-metal ions.

FUNCTION OF NICOTIANAMINE AS POSSIBLE CYTOMETALLOPHORE OF ESSENTIAL IMPORTANCE

The nicotianamine-auxotroph mutant 'chloronerva', the only vascular plant where no trace of nicotianamine has been detected, exhibits severe defects of growth and development as well as its specific type of chlorosis. Treatment with the isolated and identified "normalizing factor" nicotianamine leads to a complete restoration of the phenotype of the original tomato wild-type. This biological activity of nicotianamine (1) is very high (re-greening of leaves at 1 µM concentration, 1 nmol per seedling (ref. 21)) and approches that of phytohormones.

Alterations of the structure of 1 strongly influence its biological activity. Thus, in comparison to 1, the proline analogue 18 has an activity of only about 50 % (ref. 41), and all the other synthesized homologues and analogues of 1 (compounds 19 - 29) as well as (S)-azetidine-2-carboxylic acid (3), the corresponding dimeric compound 2, and mugineic acid (4) (ref. 22) were shown to be completely inactive as "normalizing factor". On the other hand, the also synthesized optical antipode of 1, the (+)-(R,R,R)-nicotianamine, was found to be of the same activity as the naturally occurring 1 (ref. 36).

The phenomenon of phenotypical normalization of the mutant 'chloronerva' by nicotianamine comprises numerous partial processes which are directly or indirectly induced and/or influenced by this compound. The most striking and relatively short-termed ones are (a) the re-greening of the chlorotic leaves, (b) the restoration of the disturbed ion metabolism as well as (c) the strong promotion of root development, especially the increase of root elongation.

The effects (a) and (b) seem to be strongly related. As already mentioned in the introductive chapter, the disturbed iron metabolism of the mutant is manifested by an excessive iron absorption by the roots leading to an overflow also of the leaf vessels (veins) with iron on the one hand and to an irregular cellular iron distribution within the intercostal areas of the young leaves on the other hand (ref. 3 and 4). Obviously due to its iron(II) chelating properties, nicotianamine causes or strongly promotes the intercellular transport of iron leading to an elimination of the chlorophyll defect (ref. 51). Parallel to this effect, nicotianamine (or its iron(II) complex) reduced the abnormally high iron uptake and therefore high iron content of the mutant to the levels of those of the wild-type, even after application of 1 to the leaves of the seedlings.

Important is the observation that nicotianamine controls the uptake in plants also of manganese, zinc, and copper ions by principle in the same manner as that of iron ions (ref. 22 and 53).

Despite of the fact that nicotianamine possesses no iron-solubilizing abilities and doesn't stimulate the iron uptake by rice seedlings under normal nutritional conditions (20 µM $FeCl_3$ at pH 7) (ref. 46), the amino acid 1 increases the uptake of iron by tomato and sunflower seedlings cultivated at micromolar iron concentrations (1 µM Fe-ethylenediamine N,N'-bis(2-hydroxyphenylacetic acid at pH 5) and that by supplying 1 either to the nutrient solution or to the leaves of the investigated plants. In the case of sunflower seedlings (Helianthus annuus L.) and nicotianamine application to the nutrient solution the iron uptake was significantly stimulated leading to the following figures after 4 days: 232 ± 39.1 nmol Fe/plant (205 % in comparison to the control untreated with nicotianamine) or 0.75 ± 0.11 nmol Fe/mg dry weight (147 %) (ref. 54).

In the mutant 'chloronerva' which is known for its excessive iron absorption at iron concentrations of 10 µM or above in the medium (ref. 52) the iron uptake dropped almost to zero at an iron concentration of 1 µM but showed some recovery upon nicotianamine supply, at least after its addition to the nutrient medium (ref. 54).

This apposite response of 'chloronerva' to high ($\geq$10 µM) and low (1 µM) iron supplies indicates that a nicotianamine-dependent regulation system for the iron uptake has been affected by the mutation that can be restored by addition of small amounts of nicotianamine.

The nicotianamine-auxotroph mutant 'chloronerva' is morphologically characterized among others by a severe retardation of its root system which is normalized to that of the wild-type by addition of nicotianamine to the leaves or to the nutrient solution. Since in most cases a correlation between nicotianamine (1) supply and root weight was not observed it is concluded that 1 is an effector of root elongation (ref. 51).

According to our present knowledge, nicotianamine (1) possesses an optimal structure for chelating iron(II) and other bivalent transition-metal ions. As demonstrated by the nicotianamine-auxotroph tomato mutant 'chloronerva', nicotianamine seems to be responsible for the short-distance intercellular transport of bivalent transition-metal ions and is therefore considered as a specific cytometallophore in multicellular plants. In addition to this function as carrier, nicotianamine (or one of its metal complexes) was shown to possess regulatory activities, at least in connection with the uptake of metals by plant roots. The morphogenetic effects of nicotianamine may be,

however, indirect ones caused by the restoration of the normal cellular transition-metal metabolism.

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