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ON THE "NORMALIZING FACTOR" FOR THE TOMATO MUTANT *CHLORONERVA*—XIV[1]

SYNTHESIS OF THE PROLINE ANALOGUE OF THE PHYTOSIDEROPHORE NICOTIANAMINE

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Abstract—The synthesis of (2*S*:3'*S*:3"*S*)-*N*-[3-(3-amino-3-carboxypropylamino)-3-carboxypropyl]-proline (1), a nicotianamine analogue with a 5-membered ring, was achieved by stepwise combination of *S*-proline ethyl ester (3) and (3*S*)-3-ethoxycarbonyl-3-trifluoroacetylaminopropionaldehyde (4) via reductive coupling. Compound 1 exhibits a smaller biological effect than nicotianamine (2) on phenotypical normalization of the tomato mutant *chloronerva*.

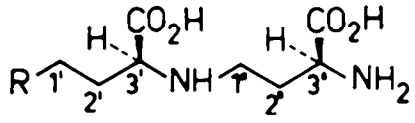
Nicotianamine (2) has been identified as the "normalizing factor" for the semi-lethal mutant *chloronerva* of the tomato, *Lycopersicon esculentum* Mill. cultivar "Bonner Beste".[2,3] Nicotianamine is an unusual non-protein amino acid which was detected[4] in many higher plants. Biochemical experiments revealed a disturbed iron metabolism of the mutant. Investigations of the complex formation with iron and other metal ions demonstrated that nicotianamine possesses an iron(II)-chelating activity.[1,4] The hitherto existing results indicate that nicotianamine is a naturally occurring chelating agent of higher plants that plays a role as a specific phytosiderophore of general importance for the cellular iron transport and/or metabolism. In connection with our investigations[5] concerning the biological action mechanism of nicotianamine (2) it was of interest to study analogues with altered ring size.

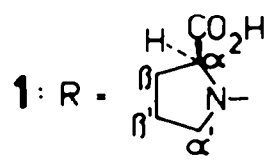
S-Proline ethyl ester (3)[6] was coupled with (3*S*) - 3 - ethoxycarbonyl - 3 - trifluoroacetylpropionaldehyde (4)[7] by catalytic hydrogenation in ethanol in the presence of palladium on charcoal to furnish (2*S*:3'*S*) - *N* - (3 - ethoxycarbonyl - 3 - trifluoroacetylaminopropyl) - proline ethyl ester (5). Alkaline or acid hydrolysis yielded (2*S*:3'*S*) - *N* - (3 - amino - 3 - carboxypropyl) - proline (6), which as triethylammonium salt was coupled once more with aldehyde 4 to give 7. Saponification of 7 with KOH yielded 1, the proline analogue of nicotianamine (2). In addition to catalytic hydrogenation reductive coupling was also carried out starting from the ethyl ester 8 and the aldehyde 4 by means of sodium cyanoborohydride at pH 6 analogously to the method used by Fushiya *et al.*[8] for the synthesis of nicotianamine (2). The reaction can be carried out in the presence of a trifluoroacetylamino group. Contrary to the Japanese workers we started the synthesis of the aldehyde component with optically active material, viz with readily available *S*-aspartic acid. Weygand and Fritz[7] published an $[\alpha]_D$ value of incorrect sign for the aldehyde 4 (see Experimental).

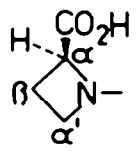
The structures of 1 and 6 were confirmed by ^{13}C NMR spectroscopy, especially by comparison with the ^{13}C-chemical shift data of proline and nicotianamine (2). The data (Table 1) exhibit the presence of thirteen C-atoms in the proton noise decoupled ^{13}C NMR spectrum of 1. The ^{13}C-chemical shifts and the off-resonance proton decoupled spectrum specified the nature of the carbons as 7 CH_2, 3 CH and 3 C=O.

The mass spectrum of 1 is different to that of nicotianamine (2). The highest peak was obtained at *m/e* 254 due to the loss of H_2O and CO_2H. Other fragments are in accordance with the expected structure (see Experimental). The IR spectrum of 1 is very similar to that of nicotianamine (2).

Compound 1 exhibits biological activity with respect to chlorophyll formation of chlorotic leaflets and root development of the mutant *chloronerva*, but to a smaller extent compared to nicotianamine (2).[9] Obviously the azetidine ring of nicotianamine is not essential for the



1: R = 

2 (nicotianamine): R = 

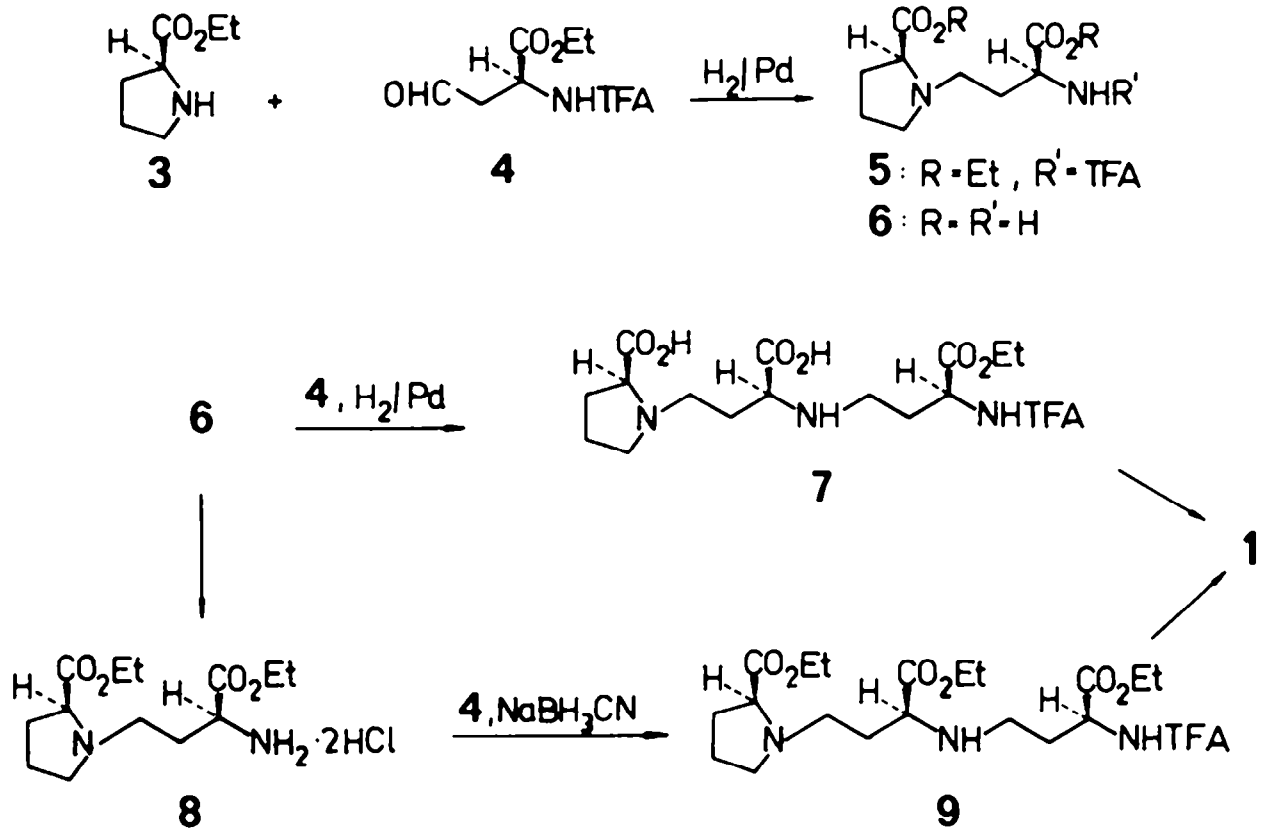

3
4
5 : R = Et , R' = TFA
6 : R = R' = H
6
4 , H2 / Pd
7
1
8
4 , NaBH3CN
9

Table 1. ^{13}C NMR spectroscopic data of 1 and related compounds in D_2O[a]

Carbon atom		1	2[3]	6	proline
	CO_2H	174.5	174.2	174.5	175.2
		173.9	174.1	173.7	-
		172.7	173.0	-	-
α-C	CH	70.2	67.7	70.3	62.0
C-3'		60.4	60.2	53.2	-
C-3"		53.5	53.4	-	-
β-C	CH_2	27.8	21.7	27.5	29.9
C-2'		26.7	25.4	29.6	-
C-2"		29.7	27.7	-	-
β'-C		23.8	-	23.7	24.6
α'-C		56.1	51.9	55.7	46.9
C-1'		52.8	51.3	53.2	-
C-1"		44.8	44.8	-	-

[a]For comparison the known effect of N-alkylation in amines (shift of ca +8 ppm in α-position, ca -3 ppm in β-position) has to be considered.

biological activity. (2*S*:3'*S*) - *N* - (3 - Amino - 3 - carboxypropyl) - proline (**6**) proved to be biologically inactive.

EXPERIMENTAL

IR spectra were measured with a Specord 75 IR (Carl Zeiss, Jena). ^{13}C NMR spectra were measured with a Bruker WP 200 spectrometer at 50.33 MHz in D_2O. Dioxane was used as an internal standard. ^{13}C-chemical shifts were recalculated for TMS using the equation: $\delta_{TMS} = \delta_{dioxane} + 67.4$ ppm. The ^1H NMR spectrum was obtained with a Varian HA 100 instrument at 100 MHz in $CDCl_3$. The δ values are in ppm downfield from HMDS. The low energy mass spectra (positive ions 10–16 eV, negative ions 2–4 eV ionization energy) were recorded on a mass spectrograph of the Research Institute "M. v. Ardenne", Dresden, G.D.R.

(3S) - 3 - *Ethoxycarbonyl* - 3 - *trifluoroacetylamino-propionaldehyde* (**4**). Synthesis according to lit.[7]; m.p. 87–88°, $[\alpha]_D^{22} = -20.5°$, $[\alpha]_{366}^{22} = -22.0°$ (abs THF, *c* 1.8), lit.[7]: m.p. 86–87°, $[\alpha]_{366}^{20} = +8.7°$ (abs THF, *c* 1.8). IR: $\nu_{max}^{KBr} = 3300$, 3100 (NH), 1743 (CO_2Et), 1717 (CHO), 1700, 1557 cm^{-1} (NHCO), ^1H NMR ($CDCl_3$): δ = 1.21 ppm (t, CH_3), 3.06 and 3.16 (m, $J_{AB} = 19$ Hz, $\underline{CH_2}$–CHO), 4.20 (q, $\underline{CH_2}$–CH_3), 4.76 (m, $J_{AX} = J_{BX} = 5$ Hz, CH), 7.47 (s, NH), 9.63 (s, CHO), MS (positive ions): *m/e* (rel. %) = 213 (74), 195 (53), 168 ($M-CO_2Et$; 98), 140 (100), (negative ions): *m/e* (rel. %) = 241 (M; 26), 240 (M-H; 31), 197 (100), 168 ($M-CO_2Et$; 70).

(2S: 3'S) - N - (3 - *Amino* - 3 - *carboxypropyl*)*proline* (**6**). A mixture of 573 mg (4 mmol) **3**, 965 mg (4 mmol) **4** and 1 g Pd/C (10% Pd) in 30 ml dry EtOH was hydrogenated at atmospheric pressure and room temp for 1 h. The resulting mixture was filtered and the solvent removed *in vacuo* to give **5** as an oil. MS (positive ions): *m/e* (rel. %) = 368 (M; 20), 323 ($M-OEt$; 43), 295 ($M-CO_2Et$; 100), 267 ($295-C_2H_4$; 43), 226 ($295-CF_3$; 84), 198 (83), 170 (77), 156 (93). Alkaline or acid hydrolysis of **5** gave the unprotected compound **6**.

(a) The residue was dissolved in 50 ml of 1% KOH-soln ($EtOH/H_2O$ = 1 : 1) and left at room temp for 20 h. The alkaline soln was given to a strongly acid cation exchanger resin (Dowex 50 WX4, 100–200 mesh, H^+-form). It was washed with distilled H_2O and eluted with 2N NH_3. Evaporation *in vacuo* gave 767 mg of crude **6**. It was purified by recrystallization from 90% EtOH or by column chromatography on Si gel (particle size 63–200 μm). The column was washed with EtOH and afterwards eluted with $EtOH/H_2O$ = 1 : 1. Silicic acid from fractions containing **6** was eliminated by filtration through a cation exchanger column (see above), washing with distilled H_2O and elution with 2N NH_3. Evaporation of the soln gave 482 mg **6** (51% yield). The white amorphous compound is pure according to TLC and was used for the next step of the synthesis without further purification.

(b) The residue was dissolved in 10 ml EtOH and heated for 30 min on the water bath with 2 × 20 ml of 18% HCl (second addition after 15 min). The reaction mixture was purified by ion exchange (see above). The resulting soln was evaporated and the remaining water was removed by repeated distillation with EtOH. To the dry residue EtOH was added and the resulting suspension was filtered. The remaining white solid (519 mg) is pure according to TLC. Evaporation of the filtrate and repetition of the hydrolysis procedure gave further 61 mg of **6**. Total yield: 580 mg (62%), crystals from $EtOH/H_2O$ = 9 : 1 as the monohydrate with dec. above 190°, $[\alpha]_D^{25} = -49.5°$ (H_2O, *c* 1.0), TLC (Si gel, n-PrOH/H_2O = 6 : 7, ninhydrin): R_f = 0.34, found: C, 46.28; H, 7.86; N, 11.85. $C_9H_{16}N_2O_4 \cdot H_2O$ requires: C, 46.15; H, 7.75; N, 11.96%. IR: $\nu_{max}^{KBr} = 1633$ cm^{-1} (CO_2^-), MS (positive ions): *m/e* (rel. %) = 198 ($M-H_2O$; 27), 181 ($M-H_2O-NH_3$; 6), 172 ($M-CO_2$; 15), 155 ($172-NH_3$; 68), 142 (54), 128 (29), 83 (100).

(2S: 3'S) - N - (3 - *Amino* - 3 - *ethoxycarbonyl*)*propyl*)*proline*

ethyl ester dihydrochloride (**8**). **8** was obtained with EtOH/HCl analogously to the preparation of **3**. The dihydrochloride (**8**) was crystaline (dec above 170°). Yield 86.5%; found: C, 44.80; H, 7.73; N, 8.04; $C_{13}H_{26}Cl_2N_2O_4$ requires: C, 45.22; H, 7.59; N, 8.11%. IR: ν_{max}^{KBr} = 1750, 1223 cm^{-1} (CO_2R).

(2S: 3'S: 3"S) - N - [3 - (3 - *Amino* - 3 - *carboxypropylamino*) - 3 - *carboxypropyl*]*proline* (**1**). (a) A mixture of 117 mg (0.5 mmol **6**, 121 mg (0.5 mmol) **4**, 0.5 ml NEt_3 and 100 mg Pd/C (10% Pd) in 30 ml MeOH was hydrogenated at atmospheric pressure and room temp for 5 h. The mixture was filtered and the catalyst washed with distilled H_2O. The solutions were combined and the solvent was removed *in vacuo* to give **7**. The removal of the protecting groups of **7** was carried out by alkaline hydrolysis. The residue was treated with 20 ml of 1N aqueous KOH at room temp for 30 min. The soln was given to a column of a cation exchanger (see above). It was washed with distilled H_2O and eluted with 2N NH_3. Evaporation of the soln gave 133 mg of crude **1**. Recrystallization from EtOH/H_2O furnished 112 mg (60%) of a white solid as the trihydrate of **1** (dec above 220°), $[\alpha]_D^{24} = -26.7°$ (H_2O, *c* 0.4); TLC (Si gel, n-PrOH/H_2O = 6 : 7, ninhydrin): R_f: 0.28; found: C, 41.91; H, 7.54; N, 11.19. $C_{11}H_{23}N_3O_6 \cdot 3H_2O$ requires: C, 42.04; H, 7.87; N, 11.32%. IR: ν_{max}^{KBr} = 1620 cm^{-1} (CO_2^-), MS (positive ions): *m/e* (rel. %) = 254 (M-H_2O-CO_2H; 16), 140 (100), 114 (proline-H; 24), 98 (*N*-ethylpyrrolidine-H; 88), 84 (*N*-methylpyrrolidine-H; 42). (b) 69 mg **8** (0.2 mmol) were dissolved in 15 ml MeOH and a soln of 22 mg (0.4 mmol) KOH in MeOH was added. The mixture was adjusted to pH 6 by HCl/MeOH and a methanolic soln of 48 mg (0.2 mmol) **4** was added. The reaction mixture was stirred for 15 min at room temp followed by addition of 9 mg $NaBH_3CN$ in MeOH. After stirring for 4 h the mixture was acidified by conc. HCl to pH < 2 and evaporated *in vacuo* to afford **9**. **9** was hydrolyzed by treatment with 10 ml 1 N aqueous KOH for 30 min and purified on a column of a cation exchanger (see above), washed with distilled H_2O and eluted with 2N NH_3. Evaporation of the soln furnished 59 mg of crude **1**. Recrystallization from EtOH/H_2O gave 49 mg (66%).

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