Tetrahedron Vol. 37, pp. 191 to 196
Pergamon Press Ltd., 1981. Printed in Great Britain

0040-4020/81/0101-0191 $02.00/0

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

MASS AND NMR SPECTROSCOPIC INVESTIGATIONS OF THE PHYTOSIDEROPHORE NICOTIANAMINE AND SOME OF ITS DERIVATIVES

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(*Received in UK 11 March 1980*)

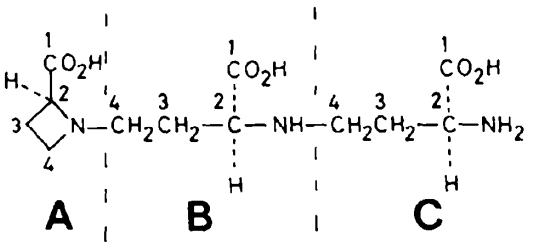
Abstract—The "normalizing factor" for the tomato mutant "*chloronerva*" was shown to possess the structure of (2*S*:3'*S*:3''*S*)-N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid (**1**) and proved to be identical with nicotianamine, especially on the basis of high resolution mass, ^1H, and ^{13}C NMR spectroscopic investigations of **1** as well as of its tetra-(trimethylsilyl), di-(4-bromobenzoyl) trimethyl ester (**3**), 4-bromobenzoyl dimethyl ester, and diacetyl methyl ester (**4**) derivatives. **1** seems to be of general occurrence in higher plants and is considered a possible phytosiderophore with an essential function in the cellular iron transport and/or metabolism.

According to recent investigations[1] the "normalizing factor" for the mutant "*chloronerva*" of the tomato, *Lycopersicon esculentum* Mill. cultivar "Bonner Beste",[2,3] was shown to possess the structure of (2*S*: 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 the unusual amino acid nicotianamine[4,5] isolated some years ago from tobacco leaves (*Nicotiana tabacum* L.)[4] and beechnuts (*Fagus silvatica* L.)[5] and detected in other plants as well.[6] Large-scale isolations[1,4,5] and screening experiments[6,7] suggested that nicotianamine (**1**) is present in all vascular plants, including fern sporophytes, but absent from non-vascular species.

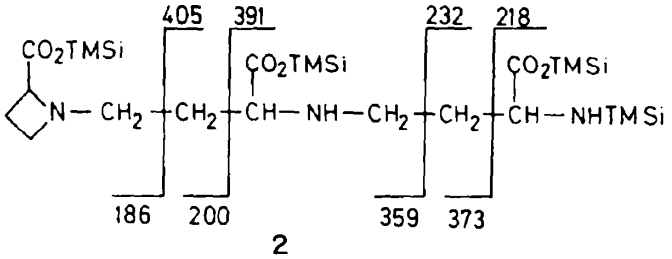
As shown from Dreiding model considerations, **1** has an optimal molecular structure for complex formation with iron(III) ions[1] which has been convincingly proved on the basis of the appearance of a positive Cotton effect at about 250 nm (pH 4.5, in H_2O).[8] According to our present knowledge, nicotianamine (**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.[1]

The structure elucidation of the "normalizing factor" for the tomato mutant "*chloronerva*" and thereafter its identification with nicotianamine (**1**) has been performed especially on the basis of high resolution mass, ^1H, and ^{13}C NMR spectroscopic investigations of **1** as well as of its tetra-(trimethylsilyl) (**2**), di-(4-bromobenzoyl) trimethyl ester (**3**), 4-bromobenzoyl dimethyl ester, and diacetyl methyl ester (**4**) derivatives.[1] The obtained results are described in detail in this paper. They seem to be of some importance not only for the identification of nicotianamine in plant material, but also for further chemical work in this field.

When mass spectrometry was used for the assessment of the elemental composition of **1**, a quasi-molecular ion ($[M + H]^+$, *m/e* 304) could only be obtained by the field desorption technique when tartaric acid was used as electrolyte. At temperatures beyond BAT *m/e* 286 and 268 (loss of one or two molecules of water from $[M + H]^+$) were observed. Even with direct inlet with electron impact (EI) only $[M - H_2O]^+$ (*m/e* 285) could be registered. Exact mass measurement of the tetra-(trimethylsilyl) derivative gave the molecular composition $C_{24}H_{53}$-



1 (Nicotianamine)



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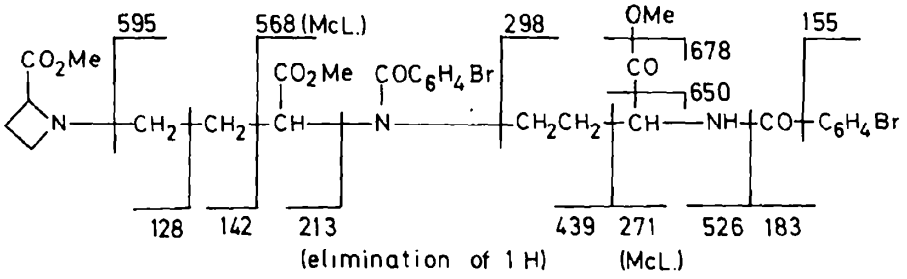

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Table 1. ^{13}C NMR data of nicotianamine and allied compounds in D_2O

Compound	Carbon (assignment)[a]											
	$-CH_2-$						$-CH-$			C=O		
Nicotianamine (1)[b]	21.7 (3A)	25.4 (3B)	27.7 (3C)	44.8 (4C)	51.3 (4B)	51.9 (4A)	53.4 (2C)	60.2 (2B)	67.7 (2A)	173.0 (1A, 1B, 1C)	174.1	174.2
Azetidine-2-carboxylic acid		24.2 (3)			43.7 (4)			59.9 (2)			174.9 (1)	
2,4-Diamino-butyric acid		32.6 (3)			37.9 (4)			54.6 (2)			180.7 (1)	

[a] The assignment of the ^{13}C signals to carbons in moieties A, B, and C was done on the basis of relative intensities (A, B, C) in the order of the expected increasing mobility of the system and on comparison with values calculated from the spectra of azetidine-2-carboxylic acid and 2,4-diaminobutyric acid by using the known effect of N-alkylation in amines (shif of ca +8 ppm in α-position, ca −3 ppm in β-position).

[b] Designation of the moieties A, B, and C as well as the numbering of atoms is according to formula 1 and not that used in chemical nomenclature.

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Table 2. ^{1}H NMR data of nicotianamine and allied compounds

Compound	Solvent	Moiety	Chemical shifts (δ)[a]					Coupling constants (Hz)							
			H-2	H-3	H-3'	H-4	H-4'	$J_{2,3}$	$J_{2,3'}$	$J_{3,3'}$	$J_{3,4}$	$J_{3,4'}$	$J_{3',4}$	$J_{3',4'}$	$J_{4,4'}$
Nicotianamine (1)[b]	D_2O	A	4.77	2.72	2.53	4.08	3.95	7.7	9.3	-11.9	4.4	9.6	9.6	9.3	-10.1
		B	3.87	2.29	2.21	3.28	3.23	7.5	5.3	-14.6	7.7	8.0	7.7	5.0	-12.6
		C	3.79	2.18	2.10	3.43	3.34	4.4	8.7	-14.4	8.1	9.1	9.1	6.1	-13.0
Azetidine-2-carboxylic acid	D_2O	-	4.80	2.75	2.53	4.09	3.92	9.8	8.2	-12.0	6.2	8.8	9.6	8.4	-10.4
2,4-Diamino-butyric acid	D_2O	-	3.43	≈1.95		≈3.03									
Nicotianamine (1)[b]	10% NaOD	A	3.54	2.23	2.09	3.22	2.80	8.9	8.9	-10.3	2.1	7.8	9.3	9.2	-7.8
		B	3.24	1.76	1.72	2.95	2.46	9.6	7.6	-13.3	6.4	10.0	10.0	5.6	-11.2
		C	3.03	1.67	1.57	2.67	2.37	5.8	7.6	-13.3	9.8	10.9	11.3	5.1	-11.3
Azetidine-2-carboxylic acid	10% NaOD	-	4.07	2.60	2.24	3.40		8.8	6.6	-11.3	8.8	7.5	7.0	7.8	?
2,4-Diamino-butyric acid	10% NaOD	-	3.26	≈1.67		≈2.63									

[a] The set of signals for moiety A follows from the ^{1}H NMR spectrum of azetidine-2-carboxylic acid. The signals of the moieties B and C were assigned tentatively by expecting a stronger effect of substitution in moiety B.

[b] Designation of the moieties A, B, and C as well as the numbering of the atoms is according to formula 1 and not that used in chemical nomenclature.

$N_3O_6Si_4$ for M^+ (*m/e* 591) and hence $C_{12}H_{21}N_3O_6$ for the "normalizing factor". This formula is in accordance with the elemental analysis of a sample dried at 180° *in vacuo* and equivalent weight determination by potentiometric titration.[1]

The IR spectrum (KBr) of **1** displays absorption at 1606 cm^{-1} (CO_2^-). The proton noise decoupled ^{13}C NMR spectrum (Table 1) proved the presence of 12 C-atoms. The ^{13}C chemical shifts and the off-resonance proton decoupled spectrum specified the nature of the carbons as $6CH_2$, 3CH and $3C{=}O$ groups. The pH dependence of the C=O signals indicated $3CO_2H$ groups (acid solution 170.8, 171.3 and 171.7 ppm, alkaline solution 181.8, 182.7, and 183.8 ppm). Since no further sp^2-hybridized carbon could be detected the compound contains one cycle. The 360 MHz ^1H NMR spectra (D_2O or 10% NaOD/D_2O) (Table 2) evidenced the presence of 15 non-exchangeable H-atoms bound to C. Hence 6 H have to be exchangeable (3 H bound to carboxyl groups, see above, and 3 H to N). Proton homonuclear selective decoupling experiments proved the existence of three isolated five-spin systems of the type $-CH_2CH_2CH\lessgtr$. Chemical shifts and coupling constants extracted by a first-order analysis were used as input values for the calculation of the theoretical spectra. The best set of calculated parameters is given in Table 2. Combination of ^1H and ^{13}C data allows the following classification: (a) $3\,CH_2$ groups are bound to C ($C{-}CH_2{-}C$): δ = 21.7, 25.4, and 27.4 ppm. (b) $3\,CH_2$ groups are bound to N ($C{-}CH_2{-}N$): δ = 44.8, 51.3, and 51.8 ppm. (c) 3 CH groups are bound to N ($C{-}CH{-}N$): δ = 53.4, 60.2, and 67.7 ppm. Hence all three sequences are of the general type

$$N{-}CH_2{-}CH_2{-}CH{-}N$$
$$\qquad\qquad\quad |$$
$$\qquad\qquad CO_2H.$$

The low field absorption of one of the five-spin systems and the relatively small absolute values of the geminal coupling constants in the ^1H NMR spectrum as compared with the other two systems (Table 2) are in accordance with the presence of a 4-membered ring. This is confirmed by comparison with the ^{13}C and ^1H NMR spectra of azetidine-2-carboxylic acid. A comparison of the ^1H signals of 2,4-diaminobutyric acid with corresponding signals of nicotianamine (**1**) indicated that the signals of the latter compound are shifted 0.2–0.4 ppm downfield. This may be due to N-substitution.

Since the MS (EI) fragmentation pattern of **1** (Fig. 1) does not correspond to the clear-cut degradation reported for the trimethyl ester,[4,5] cyclic structures have to be envisaged (*cf* also **4**) for $[M{-}H_2O]^+$ (probably of thermal origin). Hence, the interpretation can only be tentative. *m/e* 267 and 250 result from the further loss of H_2O and NH_3. *m/e* 114, 192, and 197 are obviously formed from structure **1**. *m/e* 101, 84, and 56 correspond to azetidine-carboxylic acid and its decomposition products ($M{-}OH$, $M\ CO_2H$) (*cf* 9) the series *m/e* 169, 141, and 123 may be ascribed to *a* $[a\ CO]^+$ and $[a\ CO{-}H_2O]^+$, the series *m/e* 99 and 71 to *b* and $[b{-}CO]^+$. This interpretation is in accordance with high resolution data for *m/e* 267, 250, 192, 169, 141, 99, 71, and 56.

The appearance of the chemical ionization (CI) spectra of **1** depends on the ionizing gas used. While CI (NO) due to charge exchange gives a spectrum which resembles that obtained by EI, CI (NH_3) yields by proton transfer *m/e* 268 (267 + H, *vide supra*), 250, 101 and its decomposition products as well as 99 and 71. In addition, an abundant ion (*m/e* 185) due to the loss of azetidine-carboxylic acid from *m/e* 286 (285 + H) can be observed. Similarly, in the CI spectrum (i-C_4H_{10}) *m/e* 268, 185, and 101 can be seen.

The MS fragmentation pattern of tetra-(trimethylsilyl)-nicotianamine as indicated in structure **2** follows that of nicotianamine trimethyl ester[4,5] and is in accordance with silylation of the three carboxyl groups and the primary amino group. Additional fragments correspond to $[M{-}Me]^+$ typical of TMSi compounds and losses of Me_2SiCH_2 or Me_3SiOH from various species.

Reaction of nicotianamine (**1**) with 4-bromobenzoyl chloride followed by esterification with CH_2N_2 gave two basic compounds, the bis-bromobenzoyl derivative **3** and a mono-bromobenzoyl compound. It shows a fragmentation essentially analogous to the trimethylsilyl derivative as indicated schematically (cf **3**). Several fragments can be explained readily by the operation of the McLafferty rearrangement process (McL.). *m/e* 154 and 181 can be rationalized by loss of CO_2Me and MeOH from *m/e* 213, *m/e* 385 by loss of COC_6H_4Br from *m/e* 568. The interpretation is in accordance with high resolution data for *m/e* 65[illegible] ($C_{27}H_{30}{}^{79}Br^{81}BrN_3O_6$), 213 ($C_{10}H_{15}NO_4$), and 12[illegible]

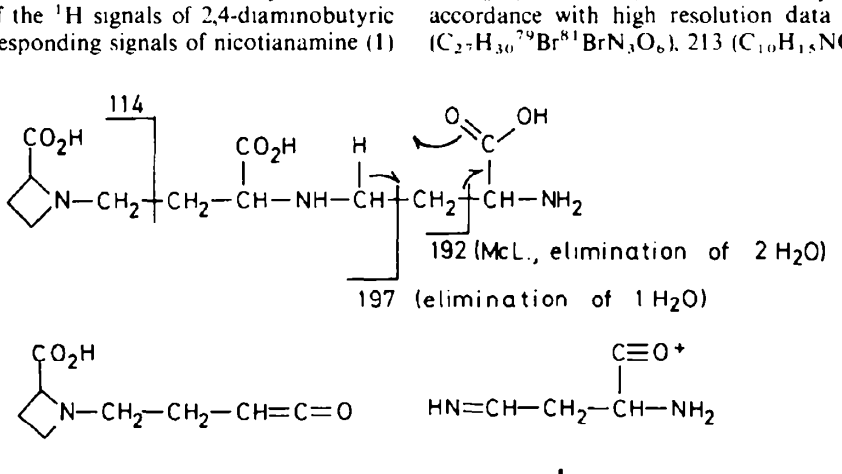

Fig. 1. MS fragmentation of nicotianamine (**1**)(EI).

($C_6H_{10}NO_2$). Most probably the mono-bromobenzoyl compound is an intramolecular γ-lactam (*cf* **4**) containing two methyl ester groups.

Reaction of nicotianamine (**1**) with Ac_2O in HOAc followed by esterification with CH_2N_2 gave a mixture, from which a diacetyl methyl ester derivative with a molecular ion $C_{17}H_{25}N_3O_7$ (*m/e* 383) was isolated. The molecular ion loses ketene, a typical behaviour of N-acetyl compounds. Obviously, during the formation of this product one molecule of H_2O has been lost. Preparation using Ac_2O-d_6 showed that two acetyl groups have been incorporated, esterification with CD_2N_2 proved the formation of one ester group and an exchange with D_2O revealed the presence of one acidic hydrogen. The fragmentation pattern differs strongly from that of nicotianamine (**1**) and from those of its derivatives discussed so far. Only two major fragments are found in the spectrum, viz. *m/e* 243 ($C_{11}H_{17}NO_5$) containing (as seen from the labelling data) one Ac group and the ester function, and *m/e* 143 ($C_6H_{11}N_2O_2$) comprising one Ac and the acidic H. Both fragments lose ketene (m*) which demonstrates the presence of NAc (rather than OAc which would be expected to lose HOAc) in either case. The fragmentation behaviour can be accommodated best, when assuming formation of a lactam, opening of the azetidine ring to a homoserine derivative and concomitant lactonization (**4**, Fig. 2). McLafferty rearrangement starting from either carbonyl group – in the case of the smaller fragment with transfer of an additional H as observed for higher alkyl amides[10] will then be responsible for the two major cleavage products. This interpretation is in accordance with high resolution data for *m/e* 383, 243, 201, 143, and 101. Structure **4** explains why the substance is non-basic.

On oxidation of nicotianamine (**1**) with performic acid a mixture was obtained from which aspartic acid was isolated (*cf* 4).

EXPERIMENTAL

^{13}C NMR spectra were measured with a Varian XL-100 instrument in FT mode at 25.2 MHz in D_2O. Dioxane was used as an internal standard. Chemical shifts were recalculated for TMS ($\delta_{dioxane}$ 67.4 ppm). ^1H NMR spectra of **1** were obtained with an FT Bruker WH-360 spectrometer at 360 MHz in D_2O or 10% NaOD/D_2O using Na 3-trimethylsilylpropionate-d_4 as an internal standard. Calculations and simulations of the spectra were done on the same instrument by an ADAKOS version 80401 programme simulation mode V-5. ^1H NMR spectra of azetidine-2-carboxylic acid and 2,4-diaminobutyric acid were measured with a Varian HA-100 instrument at 100 MHz under analogous conditions. Mass spectra were obtained with a Finnigan 3200 (compound **1**), a CH7A (Varian MAT) (**2**, **3**), or a MAT 731 (Varian-MAT) instrument (**4**; all high resolutions).

Nicotianamine (**1**). Isolated from aerial parts of alfalfa (*Medicago sativa* L.) and leaves of sugar beet (*Beta vulgaris* L.)[1], crystals with dec. above 250° and $[\alpha]_D^{20} = -49.7°$ (H_2O, *c* 1.09), lit.: −60.5° (H_2O)[4], −50° (H_2O)[5]. (After drying at 180° *in vacuo* found: C, 47.1; H, 6.9; N, 13.8. $C_{12}H_{21}N_3O_6$ requires: C, 47.5; H, 7.0; N, 13.9%.) MS (EI, 70 eV): *m/e* (rel. %): 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. %): 268 (53), 250 (30), 185 (58), 101 (18), 99 (98), 71 (52), 56 (100). MS (CI, i-C_4H_{10}): *m/e* (rel. %): 268 (47), 185 (100), 101 (32).

Tetra-(trimethylsilyl)-nicotianamine (**2**). Silylation was carried out according to[11] MS (EI, 70 eV): *m/e* (rel. %): 591 (6), 576 (3), 405 (1), 391 (2), 373 (12), 359 (6), 232 (9), 218 (7), 200 (26), 186 (60), 147 (62), 75 (77), 73 (100).

Di-(4-bromobenzoyl) trimethyl ester (**3**). **1** in N NaOH was shaken 4 days with an excess of 4-bromobenzoyl chloride. The soln was acidified, extracted with ether, NH_3 was added and the aq. layer evaporated. The residue was sorbed on Dowex 50WX8 and eluted with dilute NH_3. Treatment with CH_2N_2 at 5°C in EtOH-Et_2O and Si gel chromatography (elution with $CHCl_3$–EtOH (99:1)) afforded **3**. ν_{max}^{KBr} **3**: 3350 (NH), 1742 (CO_2Me), 1660, 1645 (N·CO), 1594 cm^{-1} (Ph). MS (EI, 100 eV): *m/e* (rel. %): 713 (1), 711 (1), 709 (1), 682 (1),

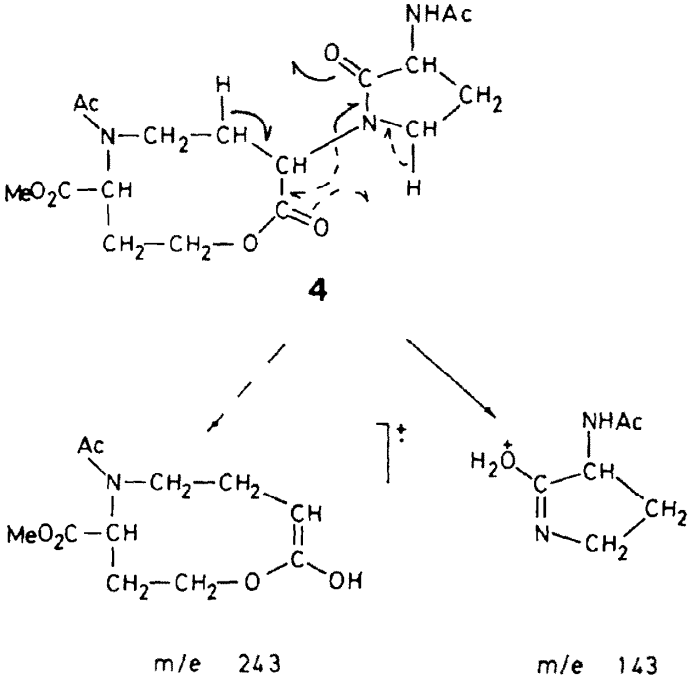

Fig. 2. MS fragmentation of the diacetyl methyl ester derivative **4** of nicotianamine (**1**).

680 (2), 678 (1), 654 (4), 652 (7), 650 (4), 599 (2), 597 (3), 595 (2), 572 (2), 570 (3), 568 (2), 528 (2), 526 (2), 441 (11), 439 (12), 387 (19), 385 (21), 300 (52), 298 (53), 273 (4), 271 (4), 213 (49), 185 (95), 183 (100), 181 (22), 157 (23), 155 (26), 154 (60), 142 (68), 128 (97).

4-*Bromobenzoyl dimethyl ester derivative*. Elution (*cf* the above mentioned chromatography) by $CHCl_3$–EtOH (49:1) gave a product with $\nu_D^{CHCl_3}$ 3: 3425 (NH), 1740 (CO_2Me), 1703 (γ-lactam), 1665 (N–CO), 1595 cm^{-1} (Ph) and MS (EI, 100 eV): *m/e* (rel. "$_o$): 497 (1), 495 (1), 465 (3), 463 (3), 438 (28), 436 (28), 383 (44), 381 (49), 356 (25), 354 (28), 185 (95), 183 (100), 142 (97), 128 (99).

Diacetyl methyl ester derivative (**4**). **1** was dissolved in HOAc–Ac_2O (1:1) by short heating. The solution was allowed to stand at room temp. for 5.5 hr, and evaporated *in vacuo*. The residue consisted of a non-basic material (no adsorption to a strongly acid sulfonated cation exchanger) and was treated with CH_2N_2 at 5°C in MeOH–Et_2O. Chromatography on Si gel and elution with $CHCl_3$–MeOH (47:3) afforded **4**. MS (EI, 70 eV): *m/e* (rel. "$_o$): 383 (72), 368 (1), 352 (3), 341 (19), 340 (14), 324 (10), 282 (36), 243 (85), 201 (100), 143 (83), 101 (67).

Oxidative degradation to aspartic acid. **1** (304 mg) was dissolved in 30 ml H_2O and a mixture of 15 ml of formic acid (98"$_o$) and 15 ml of H_2O_2 (30"$_o$) was added. After standing at 30–40° for 36 hr the mixture was evaporated in vacuo and the residue crystallized from H_2O, affording aspartic acid identical according to IR spectrum, TLC, PC, and elementa analysis with an authentic sample.

Acknowledgements—We are much indebted to Dr. K. Ubik Dr. P. Sedmera, and Dr. S. Vašíčková, Prague, for spectral measurements.

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