216. Breakdown of Chlorophyll: A Tetrapyrrolic Chlorophyll Catabolite from Senescent Rape Leaves

Preliminary Communication

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Dedicated to Prof. *Duilio Arigoni* on the occasion of his 65th birthday

(30.IX.93)

The experiments leading to the isolation and to the elucidation of the constitution of *Bn*-NCC-1, a colourless non-fluorescent chlorophyll catabolite from senescent cotyledons of rape (*Brassica napus* L.), are described. A series of fast-atom-bombardment (FAB) mass and ^{1}H- and ^{13}C-NMR spectral experiments are used to determine the constitution of the catabolite *Bn*-NCC-1. The structural information available indicates *Bn*-NCC-1 to be a 1-formyl-19-oxobilane, structurally related to 'RP 14', isolated earlier from artificially aged primary leaves of barley. The major differences between the constitution of the metal-free chlorophyll pheophorbide a and that of *Bn*-NCC-1 concern oxygenolytic opening of the porphinoid macrocycle at C(4)–C(5), saturation at the other *meso* positions, hydrolysis of the methyl-ester function, and functionalization by a malonic-acid unit of the side chain at C(8). This work provides for the first time the structural data of a chlorophyll-degradation product from senescent plant leaves formed under normal growth conditions.

Introduction. – Little is known to date of the natural fate of the chlorophylls [1] [2]. The high visibility of the phenomenon of the colouring of the autumn leaves every year again provides motivation to unravel this enigma. The massive amounts of material involved in chlorophyll breakdown on this earth (estimated to be *ca.* 10^9 t annually) [1] also demand knowledge on this subject. However, it has proven difficult to address this problem as the breakdown of chlorophyll appears to occur without leaving behind a trace [2]. A breakthrough was lately achieved in this field: in the primary leaves of barley (*Hordeum vulgare*) that were aged artificially by storage in the dark, several colourless chlorophyll catabolites could be localized *via* ^{14}C-labelling [3]; the constitution of one of these, of the nonfluorescent chlorophyll catabolite *Hv*-NCC-1 ('RP-14') could be deduced by spectroscopic means by a collaborative effort of our laboratories [4]. In related recent studies, the red pigment from the green algae *Chlorella protothecoides* [5a] was identified as a linear tetrapyrrole, apparently derived from chlorophylls by cleavage of the chlorin macrocycle at the *α-meso*-position (C(5)) also [5b, c]. On the other hand, the light-emitting substance from krill (*Euphausia pacifica*) [6a] and the dinoflagellate luciferin [6b] were discovered to be related to the chlorophylls by oxidative cleavage of the chlorin macrocycle at the *δ-meso*-position (C(20)) [6c, d].

We report here for the first time on the structure of a chlorophyll catabolite from senescent plant leaves that degreened under natural growth conditions: in the cotyledons of the dicot rape (*Brassica napus*) which aged naturally under the photoperiod with loss of their chlorophylls, a correlated accumulation of several colourless pyrrolic compounds

was recently detected [7]. The determination of the constitution of the most abundant and the most polar of these putative chlorophyll catabolites (= 'gerontophylls' [7]), of the nonfluorescent chlorophyll catabolite *Bn*-NCC-1, is the object of the present investigation.

Results and Discussion. – To obtain a uniform sample of the metabolite *Bn*-NCC-1 (see **1**), the senescent cotyledons of *Brassica napus* were cut from plants 23–25 days after sowing [7], and the cryosap obtained from 120 g of such leaves was purified by extraction and by preparative HPLC (see *Exper. Part*). Thus, 18.5 mg of the potassium salt **1** of the metabolite *Bn*-NCC-1 were obtained as a slightly yellow, powdery material[1]) (see *Exper. Part*).

The constitution of the K salt of the catabolite *Bn*-NCC-1 was deduced as that of the 1-formyl-19-oxobilane **1** from mass, ^{1}H- and ^{13}C-NMR, UV, and IR spectroscopic data (see *Exper. Part*).

The molecular formula of the K salt **1** of *Bn*-NCC-1 is $C_{37}H_{37}K_3N_4O_{11}$ as indicated by the FAB-MS [8]. The base peak appears at m/z 755.231 ± 0.02 ($C_{37}H_{40}KN_4O_{11}^+$, calc. 755.233) in the positive-mode FAB-MS, whereas the negative-ion FAB-MS exhibits a corresponding signal at m/z 753.241 ± 0.04 ($C_{37}H_{38}KN_4O_{11}^+$, calc. 753.217). The UV spectrum of **1** (neutral aqueous solution) shows a long-wavelength absorption maximum at 316 nm and is similar to that of the K salt **3** of *Hv*-NCC-1 (= 'RP-14') [4a]. In the 300-MHz ^{1}H-NMR spectrum (D_2O) of **1** the signals of all the 33 C-bound H-atoms appear (*Fig. 1*). Among these, the low-field signal CH(5)=O[2]), the spin system of a CH_2=CH group at an intermediate field, and 4 *s*'s of Me groups at high field stand out. H/D Exchange leads to disappearance of an *AB* system at 3.3 ppm (CH_2(8[5])) and of a *d* at 3.65 ppm (CH(13[2])) that couples (J = 2.2 Hz) with a signal at 4.80 ppm (CH(15))[2]). For the 37 C-centers of **1**, the ^{13}C-NMR spectrum (75 MHz, D_2O) shows 36 signals. A signal at 7.53 ppm can be assigned to 2 Me groups (C(7[1]) and C(18[1])) based on ^1J(C,H) correlations

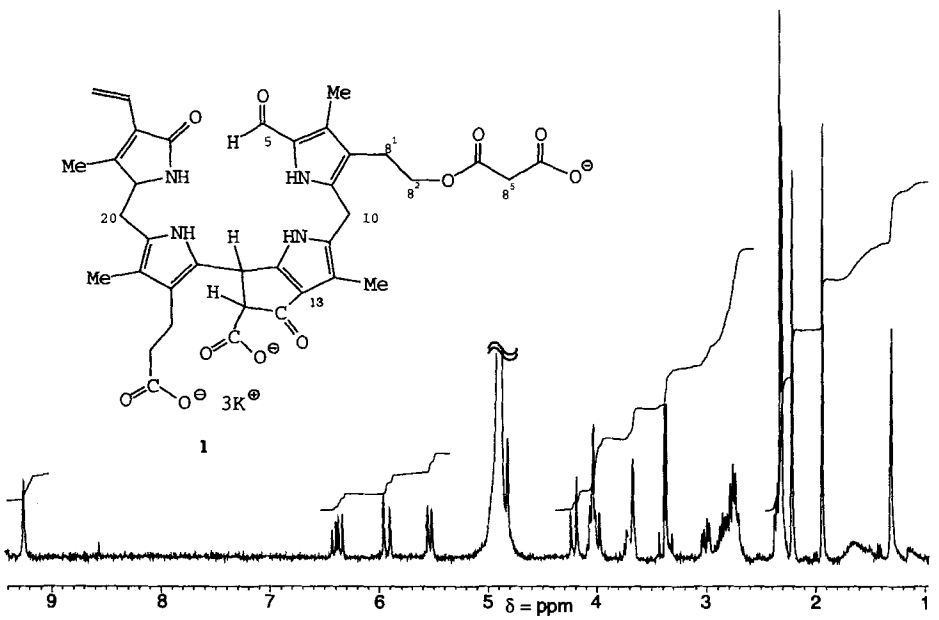


Fig. 1. *300-MHz ^{1}H-NMR Spectrum* (D_2O, 25°) *of the catabolite* Bn-*NCC-1 as its* K *salt* **1**. See text and *Exper. Part* for details.

[1]) According to a semiquantitative analysis [7], the content of *Bn*-NCC-1 in the degreened cotyledons of *Brassica napus* amounted to *ca.* 90% of the chlorophyll a (**2**) lost during senescence.

[2]) For convenience, the numbering system used for **1** corresponds to that of chlorophyll a (**2**) [9].

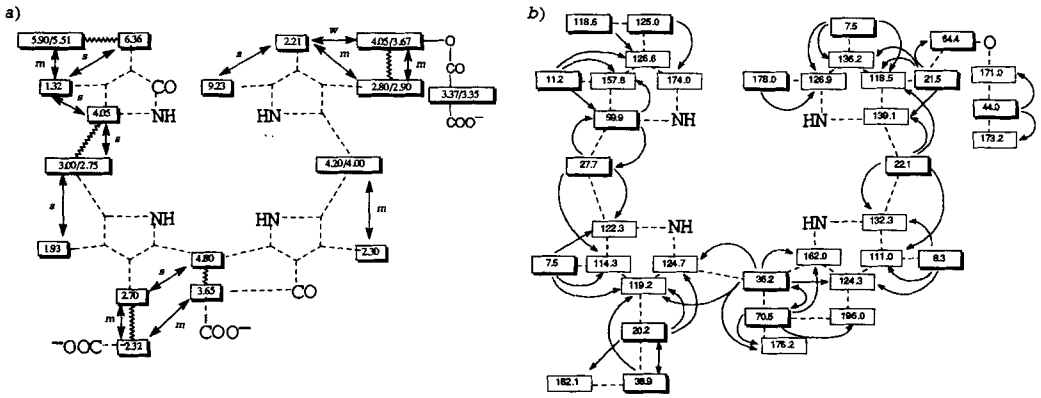


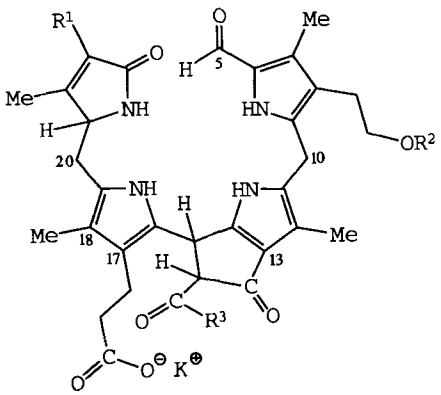
Fig. 2. a) *Assignment of the ^{1}H-NMR signals of* **1** *in D_2O* (25°C, N_2), *NOE-correlations* (arrows) *from the ROESY spectrum* (*w* = weak, *m* = medium, *s* = strong) *and J(H,H)-correlations* (wavy lines) *from COSY spectrum.* b) *Assignment of the ^{13}C-NMR signals of* **1** *in D_2O* (25°C, N_2) *and ^{2}J(C,H)/^{3}J(C,H) correlations* (arrows pointing from ^{1}H site to correlated ^{13}C). NH(24) shows additional ^2J(C,H)/^3J(C,H) correlations to all 4 C-atoms of the pyrrole ring D.

to 2 *s*, at high field in the ^{1}H-NMR. With the help of homonuclear spectra (phase-sensitive DQF-COSY) [10] and NOE experiments (ROESY [10b] [11]) as well as of heteronuclear ^1J(C,H) and ^2J(C,H)/^3J(C,H) correlations (HMQC [10b] [12] and HMBC spectra [10b] [13]), the connectivity of the chlorin-bound H-atoms and of the C-atoms of the tetrapyrrole skeleton of **1** are established (see *Fig. 2*). The bonds to N(24) are derived analogously. The positions of the other 3 N-atoms are assigned in agreement with an analysis of the ^{13}C-NMR chemical shifts of the pyrrole-ring C-atoms. Of the 11 O-atoms of **1**, 7 are assigned to C=O and COO groups, whereas the 4 remaining O-atoms are assigned to the (KOOC) CH_2COO moiety appended at ring *B*. The point of attachment of this side chain can not be derived directly (from HMBC spectra); however, the ^{1}H- and ^{13}C-NMR chemical shifts of $CH_2(8^1)$ and $CH_2(8^2)^2$) are characteristic of an acyloxy substituent at C(8^2). The presence of such an appended potassium-malonate functionality is not only compatible with the IR spectra, but is required to explain the presence of a CH_2 unit bearing diastereotopic protons (*AB* system) that exhibit ^2J(C,H) correlations to the C-atoms of an ester and of a carboxyl group.

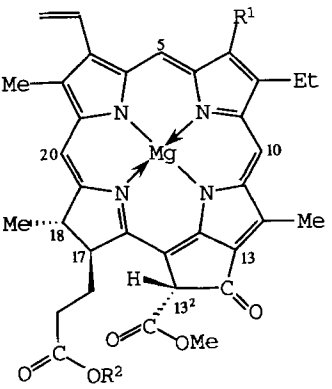
The analysis of all the spectroscopic data indicates that the K salt **1** of the catabolite *Bn*-NCC-1 has the constitution of a *tripotassium 13^2-carboxylato-8^2-(carboxylatoacetoxy)-3^1,3^2-didehydro-1,4,5,10,15,20,22,24-octahydro-4,5-dioxo-4,5-seco-21*H,*23*H-*phytoporphyrinate* [9][2]), *i.e.* of a linear tetrapyrrole with the backbone structure of a 1-formyl-19-oxobilane [14]. The configuration of the three stereogenic centers of *Bn*-NCC-1 (C(1), C(13^2), and C(15^2)) has not yet been established; however, tetrapyrrole **1** is indicated by the NMR spectra to be a single diastereoisomer.

The constitution of the K salt **1** of the catabolite *Bn*-NCC-1 from the dicot rape (*Brassica napus*) was deduced here using modern spectroscopic techniques. The basic constitution of **1** is identical to that of the K salt **3** of the catabolite 'RP-14' (*Hv*-NCC-1) [4], found in the vacuoles of artificially aged leaves of the monocot barley (*Hordeum vulgare*) [3]. The structure of these two colourless, linear tetrapyrroles indicate that they are formed from chlorophyll a (**2**) *via* chlorophyllide a (**4**) or its metal-free ligand, pheophorbide a, rather than from chlorophyll b (**5**)[3]). In **1** and **3**, the chromophoric

[3]) A catabolite that can rationally be derived from chlorophyll b (CHO group at C(7)) was recently found in cultures of the green algae *Chlorella protothecoides* in *Gossauer*'s laboratory [15], but not yet in senescent plants. We thank Prof. Dr. *A. Gossauer*, University of Fribourg, for communicating these results to us prior to publication.



1 $R^1 = CH_2{=}CH$, $R^2 = (KOOC)CH_2CO$, $R^3 = OK$, K salt of *Bn*-NCC-1
3 $R^1 = CH_2(OH)CH(OH)$, $R^2 = H$, $R^3 = MeO$, K salt of *Hw*-NCC-1



2 $R^1 = Me$, $R^2 =$ phytyl, chlorophyll a
4 $R^1 = Me$, $R^2 = H$, chlorophyllide a
5 $R^1 = CHO$, $R^2 =$ phytyl, chlorophyll b

system of their precursor pigment **2** is broken up into colourless units, apparently by an (oxidative) opening of the chlorin macrocycle at the *α-meso*-position (C(4)–C(5)) and by saturation of the remaining *meso*-bridges. A series of polar functionalities, notably a malonyl unit[4]) in **1**, increase the hydrophilicity of **1** and of **3**.

The related findings on the structure of **1** and of **3** suggest a common basic pattern of chlorophyll breakdown in senescent plant material, apparently comparable also to that in some green algae [5]. The crucial step of the enzymatic degradation of the green plant pigment appears to be an (oxidative) ring opening at C(4)–C(5) without loss of a C-unit, contrasting earlier proposals, based on model chemistry [1]. This enzyme-catalyzed ring cleavage presumably takes place to rapidly suppress the potentially harmful photodynamic behaviour of the chlorophylls [1–3] in senescent parts of green plants.

We would like to thank Doz. Dr. *E. Müller* and Dr. *R. Konrat*, University of Innsbruck, for their help with the NMR spectra, Dr. *D. Moskau*, *Bruker-Spectrospin*, Fällanden, for high-field 2D-NMR spectra, and Dr. *W. Amrein*, *R. Häfliger*, and *O. Greter*, Laboratory of Organic Chemistry, ETH-Zürich, for acquiring mass-spectral data. Work in Zürich was supported by the *Swiss National Science Foundation*, work in Innsbruck by the University of Innsbruck.

Experimental Part

1. *General.* Acetone and hexane (both *puriss.*, *p.a.*) from *Fluka*, Buchs, *Sep-Pak-C_{18}* cartridges from *Waters Assoc.*, Milford, USA. UV/VIS (H_2O): *Hitachi U-3000*; λ_{max} in nm (rel. int. in %). (FT)-IR (0.2% in KBr): *Mattson 3000*; ν[cm^{-1}]. ^{1}H-NMR (300.13 MHz, D_2O): *Bruker AM-300*; δ(HDO) 4.90 ppm at 23°. ^{13}C-NMR (75.469 MHz, D_2O): *Bruker AM-300*. FAB-MS (cesium bombardment at 35 keV): *ZAB-2 SEQ*; *m/z* (%).

2. *Isolation of the Catabolite.* The cultivation of the rape plant (*Brassica napus* L.) was carried out as described in [7]. The senescent cotyledons of *Brassica napus* were harvested 23–25 days after sowing and worked up in 12 portions of 10 g each. The cryosap (*ca.* 8 ml) from each portion was treated with acetone (*ca.* 32 ml) to separate most of the proteins by centrifugation (5 min/2300 × *g*). To the supernatant, hexane (*ca.* 40 ml) was added, the mixture shaken, and the aq. phase divided into 4 portions to be purified further by HPLC (20 × 250 mm column, *ODS-Hypersil RP18*, 5 μm; MeOH/100 mM K-phosphate buffer (pH 7) 4:6 (*o/o*), flow rate 10.25 ml/min;

[4]) See *e.g.* [16].

detection at 320 nm). The fractions with a retention time of 20.4 min were collected under N_2 at 0° and lyophilized. The residues were combined in distilled H_2O/(25 ml) and desalted on 2 *Waters-C_{18}-'Sep-Pak'* cartridges. With distilled H_2O (40 ml), **1** was eluted as a slightly yellow soln. which was lyophilized at $T < 0°$. The residue was dried at r.t. in high vacuum. From 120 g of degreened cotyledons, *ca.* 18.5 mg of slightly yellow, powdery K salt **1** of *Bn*-NCC-1 were obtained.

3. *Selected Spectroscopic Data of* **1**. UV/VIS ($c \approx 3 \cdot 10^{-5}$ M) 244 (100), 314 (91). IR (0.2% in KBr): 1719*m*, 1670*s*, 1636*s*, 1595*s*,1385*m*, 1083*s*, 986*m*. ^{1}H-NMR (300 MHz): 1.32, 1.93, 2.21, 2.30 (4*s*, Me(2^1), Me(18^1), Me(7^1), Me(12^1)); 2.32 (*m*, $CH_2(17^2)$); 2.70–2.90 (*m*, $CH_2(17^1)$, $CH_2(8^1)$, H_a–C(20)); 3.00 (*dd*, J = 5.3, 14.3, H_b–C(20)); 3.35, 3.39 (*AB*, J = 20.3, $CH_2(8^5)$); 3.65 (*d*, J = 2.2, H–C(13^2)), superimposed by 3.67 (*m*, H_a–C(8^2)); 4.00, 4.20 (*AB*, J = 16.8, CH_2(10)); 4.05 (*dd*, J = 5.3, 7.8, H–C(1)), superimposed by 4.05 (*m*, H_b–C(8^2)); 4.80 (*d*, J = 2.2, H–C(15)); 5.51 (*dd*, J = 1.2, 11.9, H_a–C(3^2)); 5.90 (*dd*, J = 1.2, 18.2, H_b–C(3^2)); 6.36 (*dd*, J = 11.9, 18.2, H–C(3^1)); 9.22 (*s*, H–C(5)). ^{13}C-NMR (75 MHz): 7.53 (C(7^1), C(18^1)); 8.30 (C(12^1)); 11.2 (C(2^1)); 20.2 (C(17^1)); 21.5 (C(8^1)); 22.1 (C(10)); 27.7 (C(20)); 36.2 (C(15)); 39.0 (C(17^2)); 43.9 (C(8^5)); 59.9 (C(1)); 64.4 (C(8^2)); 70.5 (C(13^2)); 111.0 (C(12)); 114.3 (C(18)); 118.5 (C(8)); 118.6 (C(3^2)); 119.2 (C(17)); 122.3 (C(19)); 124.3 (C(13)); 124.7 (C(16)); 125.0 (C(3^1)); 126.6 (C(3)); 126.9 (C(6)); 132.3 (C(11)); 136.2 (C(7)); 139.1 (C(9)); 157.8 (C(2)); 162.0 (C(14)); 171.1 (C(8^4)); 173.2 (C(8^6)); 174.0 (C(4)); 176.2 (C(13^3)); 178.0 (C(5)); 182.1 (C(17^3)); 196.0 (C(13^1)). FAB-MS: positive-ion mode (matrix: 3-nitrobenzyl alcohol): 869.2 (25, $C_{37}H_{37}K_4N_4O_{11}^+$, $[M + K]^+$), 832.1 (24), 831.1 (65, $C_{37}H_{38}K_3N_4O_{11}^+$, $[M + 1]^+$), 795.2 (25), 794.2 (43), 793.2 (86, $C_{37}H_{39}K_2N_4O_{11}^+$, $[M + 2 - K]^+$), 757.2 (20), 756.2 (42), 755.231 ± 0.02 (73, $C_{37}H_{40}KN_4O_{11}^+$, $[M + 3 - 2K]^+$, calc. 755.233), 717.2604 (24, $C_{37}H_{41}N_4O_{11}^+$, $[M + 4 - 3K]^+$, calc. 717.2771); 711.2 (42, $C_{36}H_{40}KN_4O_9^+$, $[M + 3 - 2K - CO_2]^+$), 591.2 (40), 590.2 (44), 589.2 (50), 588.2 (100, $C_{29}H_{31}KN_3O_8^+$, $[M + 3 - 2K - CO_2 - \text{ring A}]^+$), 552.2 (44), 551.2 (57), 550.2 (74, $C_{29}H_{32}N_3O_8^+$, $[M + 4 - 3K - CO_2 - \text{ring A}]^+$), negative-ion mode (matrix: glycerine): 792.2 (8), 791.2 (19, $C_{37}H_{37}K_2N_4O_{11}^-$ $[M - K]^-$), 755.2 (10), 754.2 (24), 753.241 ± 0.04 (100, $C_{37}H_{38}KN_4O_{11}^-$, $[M + 1 - 2K]^-$, calc. 753.217), 717.2 (9), 716.2 (41), 715.262 (51, $C_{37}H_{39}N_4O_{11}^-$, $[M + 2 - 3K]^-$, calc. 715.262), 666.2 (10), 665.2 (24, $C_{35}H_{38}KN_4O_7^-$, $[M + 1 - 2K - 2CO_2]^-$), 629.2 (15), 628.2 (16) 627.2 (41, $C_{35}H_{39}N_4O_7^-$, $[M + 2 - 3K - 2CO_2]^-$), 542.1 (13, $C_{28}H_{29}KN_3O_6^-$, $[M + 1 - 2K - 2CO_2 - \text{ring A}]^-$), 506.2 (11), 505.2 (41), 504.2 (43, $C_{28}H_{30}N_3O_6^-$, $[M + 2 - 3K - 2CO_2 - \text{ring A}]^-$).

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