Breakdown of Chlorophyll: A Fluorescent Chlorophyll Catabolite from Sweet Pepper (*Capsicum annuum*)

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The primary fluorescent chlorophyll catabolite **1** (*Ca*-FCC-2) from sweet pepper (*Capsicum annuum*) has similar optical properties, but is slightly less polar than the primary FCC (pFCC; **2**) from senescent cotyledons of oilseed rape (*Brassica napus*). *Ca*-FCC-2 was prepared from pheophorbide a using an enzyme extract from ripe *C. annuum* chromoplasts. The catabolite *Ca*-FCC-2 (**1**) could be determined from fast-atom-bombardment (FAB) mass spectra to be an isomer of pFCC (**2**). The constitution of *Ca*-FCC-2 was determined by homo- and heteronuclear magnetic-resonance experiments and was found to be identical to that of pFCC. Further 2D-homonuclear spectra of *Ca*-FCC-2 revealed it to differ from pFCC by the configuration at the methine atom C(1), whose configuration results from the action of red chlorophyll catabolite reductase (RCCR). The occurrence of two primary FCCs that are epimeric at C(1) provides a structural basis for the recent observation of two types of RCCRs among higher plants.

Introduction. – The enzymatic degradation of chlorophylls in senescent higher plants rapidly progresses to colorless tetrapyrrolic chlorophyll catabolites [1][2] that are found in the vacuoles of the degreened parts of the plant [3]. The chlorophyll catabolites from higher plants have, therefore, long remained undiscovered, and chlorophyll breakdown was elusive until this decade [4b]. In the meantime, our knowledge on chlorophyll catabolism has considerably advanced [1][5][6].

When the rate of chlorophyll breakdown in senescing plants is high, traces of fluorescing compounds were detected that were suggested to represent fluorescent chlorophyll catabolites (FCCs) and reactive intermediates in the conversion of chlorophyll to its colorless degradation products [1][7]. With an enzyme extract obtained from degreened cotyledons of oilseed rape (*Brassica napus*), a sample of the primary FCC (pFCC = *Bn*-FCC-2) was prepared and identified analytically with an *ex vivo* FCC from oilseed rape. The constitution of *Bn*-FCC-2 (pFCC) was delineated with spectroscopic methods as a 1-formyl-19-oxobilane derivative [8]. The structure of pFCC suggested that it was derived from pheophorbide a by oxidative cleavage of the tetrapyrrole macrocycle at the C(4)–C(5) positions[1]) and saturation at the C(10) and C(20) *meso* positions. Consistent with this, pFCC was found to arise *in vitro* from pheophorbide a by the joint action of two enzymes, pheophorbide-a oxygenase (PaO) and red-chlorophyll-catabolite reductase (RCCR) [1][9][10]. The membrane-bound

[1]) As had consistently been used earlier [1][2], the atoms of the tetrapyrroles are numbered according to the *IUPAC* recommendations for chlorophylls (see, *e.g.*, [4a]).

PaO attaches one O-atom of molecular oxygen to pheophorbide a [11] and produces a tightly bound form of the red chlorophyll catabolite (RCC). In an *in vitro* assay with pheophorbide a and a membrane extract from gerontoplasts of oilseed rape, small quantities of unbound RCC were obtained [9] that was structurally characterized by identification with authentic RCC prepared by partial synthesis from pheophorbide a [12]. RCC is similar in its structure to chlorophyll catabolites produced and excreted by the green alga *Chlorella protothecoides* when grown under deficiency of nitrogen [6][13]. Due to metabolic cooperation between PaO and RCCR, in gerontoplasts, the PaO-bound RCC is immediately reduced *in vivo* at the C(1)=C(20)[1] bond by RCCR to the fluorescing chlorophyll catabolite pFCC [8]. pFCC, therefore, is the primary catabolite to accumulate in noticeable amounts *in vivo* during chlorophyll breakdown in oilseed rape (*Fig. 1*) [1][8].

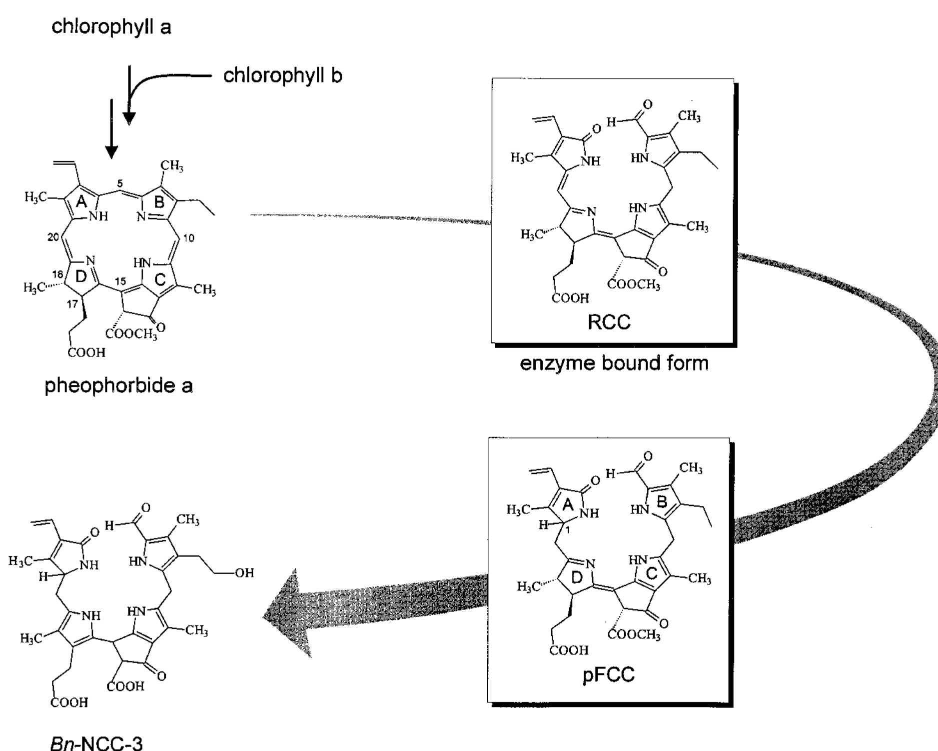

Fig. 1. *Structural outline of key steps in chlorophyll breakdown in senescent cotyledons of oilseed rape* [1]

Two major types of primary FCCs appear to be formed in the plant kingdom, pFCC (*Bn*-FCC-2; **2**) and a further, less polar primary FCC (*Ca*-FCC-2; **1**). The two primary FCCs can be distinguished by their different retention times on a reversed-phase HPLC column. The natural source of the reducing enzyme RCCR has been suggested to be responsible for the type of primary FCC produced [10]. Accordingly, two types of red-chlorophyll-catabolite reductases (RCCR), provisionally named RCCR(type 1) and

RCCR(type 2), appear to have evolved in higher plants [14]. To investigate the biochemistry of RCCR(type 1) and RCCR(type 2), information on the molecular structure of their reaction products (primary FCCs or pFCCs) and on their structural relationship is essential.

The less polar primary FCC **1** (*Ca*-FCC-2) is found in sweet pepper (*Capsicum annuum*) [15]. Here we report an *in vitro* synthesis of **1**, using an enzyme preparation from *C. annuum*, the elucidation of the constitution of **1**, and the delineation of its structural relationship with *Bn*-FCC-2 (pFCC; **2**).

Results and Discussion. – The fluorescent chlorophyll catabolite *Ca*-FCC-2 (**1**) was prepared *in vitro* from pheophorbide a using a procedure analogous to that reported earlier for the preparation of pFCC (**2**) [8]. The UV/VIS spectrum of **1** showed absorption maxima at 318 and 358 nm, similar to that of **2**. Likewise, the fluorescence spectrum of **1** exhibited a maximum at *ca*. 440 nm, similar to that of **2**. The CD spectra of the two FCCs differ mainly by an apparent sign change of one band at *ca*. 280 nm. The molecular formula of **1** was deduced from the determination of the exact mass using fast-atom-bombardment (FAB) as ionization mode [16]. The signal of the pseudo-molecular ion ($[M+H]^+$) was found at m/z 629.2960 ± 0.005 Da, corresponding to a molecular formula of $C_{35}H_{40}N_4O_7$ (calc. 629.2975). The same molecular formula was found for *Bn*-FCC-2 (**2**) [8], indicating **1** to be an isomer of **2**.

The constitution of *Ca*-FCC-2 was delineated with homo- and heteronuclear NMR-spectroscopic methods. Signals for all 36 C-bound protons were found in the 500-MHz ^{1}H-NMR spectrum of **1** (see *Table*). Measurements were carried out at −20° and under inert atmosphere, again [8] to prevent decomposition of the sample and to slow down H/D exchange of the signal at 4.51 ppm, which disappeared within two days. A signal of a formyl group (9.35 ppm), signals of the coupled protons of a vinyl and an ethyl, and four uncoupled Me groups (4 *s*) were identified, one of them being an ester Me group according to its chemical shift. A fifth Me group (*d* at 1.14 ppm) was assigned to Me (18^1)[1]. Six spin systems could be identified in the molecule using homonuclear 2D experiments (DQF-COSY, TOCSY) [17] (*Fig. 2*). ^{1}H,^{1}H-Correlations through space from ROESY spectra [17] allowed the determination of the sequence of substituents on the tetrapyrrolic 'macrocycle' and to define unambiguously C(4)−C(5)[1] as the position of the oxidative cleavage of the porphyrin macrocycle. From these data and from further studies by ^{1}H,^{13}C-heteronuclear experiments (HSQC), the fluorescent **1** was assigned the structure of a 3^1,3^2-didehydro-1,4,5,10,17,18,20,22-octahydro-13^2-(methoxycarbonyl)-4,5-dioxo-4,5-secophytoporphyrin. Accordingly, the two primary FCCs, **1** and **2**, from higher plants have identical constitution.

The linear tetrapyrrole **1** represents an optically active 1-formyl-19-oxobilane with four stereogenic centers of uniform configuration. According to the ROESY experiments, the relative configuration at the chiral centers 13^2, 17, and 18 is identical to that in pheophorbide a and hence also (in all likelihood) their absolute configuration would be the same as in pheophorbide a. The same conclusion was reached earlier when delineating the structure of pFCC (see *Fig. 2*) [8]. The configurational identity with respect to the chiral centers at 13^2, 17, and 18 and with regard to the chromophoric system across the rings C and D would also be consistent with the similarity of the long-wavelength parts of the CD spectra of **1** and **2** (see [8] and *Exper. Part*).

Table 1. *Signal Assignments for 500-MHz ^{1}H-NMR and 125-MHz ^{13}C-NMR Spectra* (CD_3OD, $-20°$) *of* Ca-*FCC-2* (**1**) *and of* Bn-*FCC-2* (pFCC; **2**). ^{13}C-Assignments from HSQC spectra.

	Ca-FCC-2 (**1**)			*Bn*-FCC-2 (pFCC, **2**)		
	δ(H) [ppm]	δ(C) [ppm]	Multiplicity, coupling constant [Hz]	δ(H) [ppm]	δ(C) [ppm]	Multiplicity, coupling constant [Hz]
$Me(8^2)$	0.99	16.0	*t*, $J = 7.5$	0.98	14.8	*t*, $J = 7.7$
$Me(18^1)$	1.14	19.0	*d*, $J = 7.2$	1.13	17.8	*d*, $J = 7.2$
$H_a-C(17^1)$	1.64	30.2	*m*	1.70	30.0	*m*
$H_b-C(17^1)$	1.94	30.2	*m*	1.96	30.0	*m*
$CH_2(17^2)$	2.23	34.0	*m*	2.09	27.7	*m*
$Me(2^1)$	2.10	9.5	*s*	2.10	11.9	*s*
$Me(12^1)$	2.14	9.4	*s*	2.13	8.8	*s*
$Me(7^1)$	2.26	9.5	*s*	2.25	8.3	*s*
$H-C(17)$	2.38	48.7	*m*	2.38	47.9	*m*
$CH_2(8^1)$	2.44	18.5	*q*, $J = 7.5$	2.40	17.2	*q*, $J = 7.7$
$H_a-C(20)$	2.62	35.2	*AMX*, $J = 18.1, 7.8$	2.53	34.4	*AMX*, $J = 18.0, 8.5$
$H_b-C(20)$	3.10	35.2	*AMX*, $J = 18.1, 4.0$	3.06	34.4	*AMX*, $J = 18.0, 3.4$
$H-C(18)$	2.70	54.5	*qd*, $J = 7.2, < 1$	2.79	51.1	*qd*, $J = 7.2, 1.7$
$Me(13^5)$	3.75	54.4	*s*	3.77	52.8	*s*
$H_a-C(10)$	4.02	23.6	*AB*, $J = 17.4$	3.99	23.0	*AB*, $J = 16.4$
$H_b-C(10)$	4.02	23.6	*AB*, $J = 17.4$	4.03	23.0	*AB*, $J = 16.4$
$H-C(1)$	4.58	59.8	*AMX*, $J = 7.8, 4.0$	4.63	57.9	*AMX*, $J = 8.5, 3.4$
$H-C(13^2)$	4.51	63.0	*s*	4.62		*s*
$H_{trans}-C(3^2)$	5.39		*dd*, $J = 12.0, 2.0$	5.41	119.0	*dd*, $J = 12.8, 2.2$
$H_{cis}-C(3^2)$	6.26		*dd*, $J = 17.5, 2.0$	6.27	119.0	*dd*, $J = 17.1, 2.2$
$H-C(3^1)$	6.53		*dd*, $J = 17.5, 12.0$	6.56	127.1	*dd*, $J = 17.1, 12.8$
$H-C(5)$	9.35		*s*	9.33	177.5	*s*

The available information, therefore, suggested that **1** and **2** would be stereoisomers at C(1). This provisional conclusion was corroborated by further NMR studies. NOE Correlations show significant differences between **1** and **2** only in the A/D part of the two isomeric molecules. A long-range NOE between $Me(2^1)$ and $Me(18^1)$ in *Ca*-FCC-2 (**1**) is not visible in the ^{1}H,^{1}H-ROESY of **2**[2]). Another significant difference concerns the homonuclear ^{1}H,^{1}H-correlation (ROESY) between $Me(18^1)$ and $CH_2(20)$, **2** exhibiting an NOE between $Me(18^1)$ and the highfield proton of $CH_2(20)$ [8] and **1** between $Me(18^1)$ and the downfield proton of $CH_2(20)$ (see *Fig. 2*). These differences in the ROESY correlations also support a configurational difference between the two primary FCCs, which then has to arise from the absolute (and relative) configuration at C(1). Accordingly, *Ca*-FCC-2 (**1**) is suggested to be the 1-epimer of pFCC (**2**), thus 1-*epi*-pFCC (see *Figs. 1* and *3*). However, at this stage, the absolute configuration at C(1) of neither of these two primary FCCs can be assigned with confidence.

The conversion of pheophorbide a to primary FCCs is catalyzed by the joint action of the two enzymes PaO, a membrane-bound enzyme, and the soluble stroma enzyme RCCR [1] [9]. The former cleaves the porphyrin macrocycle oxidatively and produces a bound form of the intermediary catabolite (RCC) [12], which seems to be reduced stereoselectively on the C(20)=C(1)[1]) bond by the action of the reductase (see *Fig. 4*).

[2]) Fig. 2 (left side) of the report on pFCC (*Bn*-FCC-2; **2**) [8] erroneously exhibits an arrow indicating the observation of an NOE between $Me(18^1)$ and $Me(2^1)$.

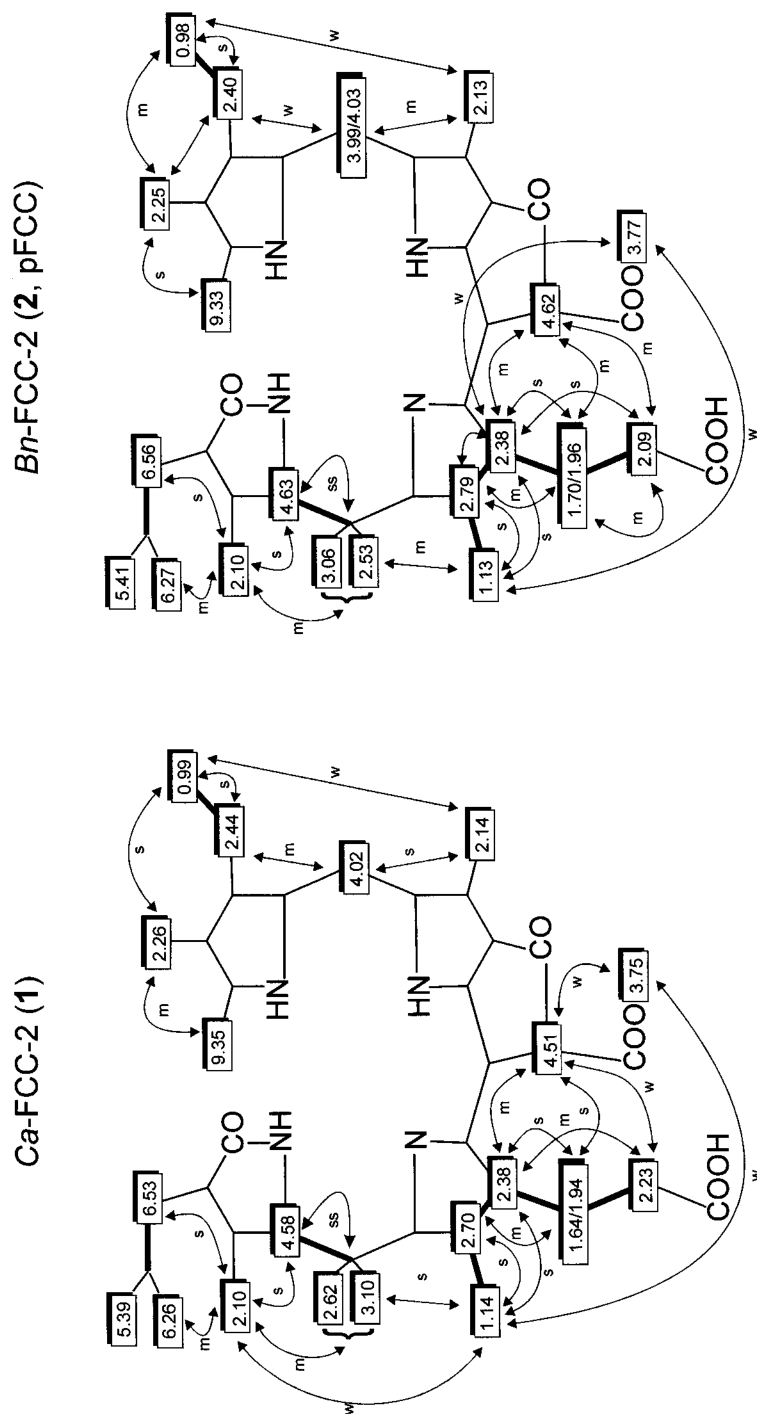

Fig. 2. *Signal assignments for the 500-MHz* ^1H*-NMR spectrum* a) *of* Ca-FCC-2 (**1**) *and* b) *of* Bn-FCC-2 (**2**) *in* CD_3OD*, as well as* $^1H,^1H$*-correlations from COSY* (solid line) *and ROESY experiments* (double headed arrows). Abbreviations: s: strong, ss: very strong, m: medium, and w: weak

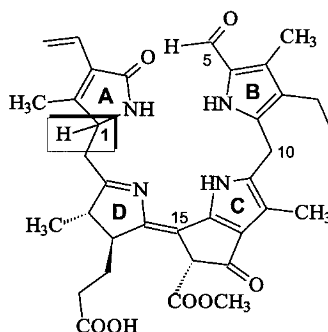

Fig. 3. *Common constitutional formulae of* Ca-*FCC-2* (**1**) *and* Bn-*FCC-2* (pFCC; **2**), *which are epimeric at C(1)*

The work described here revealed the two primary FCCs found in two senescent plant species to differ from each other by the configuration at C(1). This finding is consistent with the suggestion that the type of primary FCC formed is determined exclusively by the (source of the) RCCR [10]. Screening of 62 plant species for their primary FCCs showed that a single isomer was produced from each species and that the type of reduction product was uniform within families, as was, at the same time, the type of RCCR expressed. It also revealed that RCCR(type 2) is predominant, whereas RCCR(type 1) seems to represent a recent derivation, which in unrelated lineages has evolved independently from RCCR(type 2) [18].

Products of the oxidative cleavage of the chlorophyll macrocycle have been identified in vascular plants [1][2] [19–22] as well as in the green alga *Chlorella protothecoides* [6][13b][23]. Whereas the alga excretes red chlorophyll catabolites into the culture medium, in higher plants, chlorophyll is degraded into polar, colorless catabolites [1][2], the so called NCCs (nonfluorescing chlorophyll catabolites), which are stored in the vacuole [3]. Interestingly enough, the red bilins excreted by *C. protothecoides* have the same basic tetrapyrrole constitution as RCC [12]. Accordingly, the red catabolite RCC, which is found (in traces) as isolate from PaO-catalyzed ring opening of pheophorbide a in higher plants [10], is presumed to represent a key intermediate during chlorophyll breakdown. Likewise, in both the green alga [23] and higher plants [11], the ring-cleaving enzymes are indicated to be monooxygenases (from incorporation of one O-atom from O_2 at C(5)). Aside from a more stringent (apparent) substrate specificity of PaO (the enzyme in the higher plants [24]), the available information is consistent with similar basic mechanisms of macrocycle cleavage in both organisms [11][23].

However, chlorophyll catabolism in higher plants differs remarkably from that in the green alga by the formation of FCCs and NCCs [1][6]. The primary FCCs are rapidly formed from pheophorbide a (in a metabolically channeled way) by the joint action of the O_2-dependent PaO and the O_2-sensitive RCCR. The role of the RCCRs in higher plants is of interest with regard to the evolution of chlorophyll breakdown: since they are present in higher plants, but absent from *Chlorella protothecoides* [18],

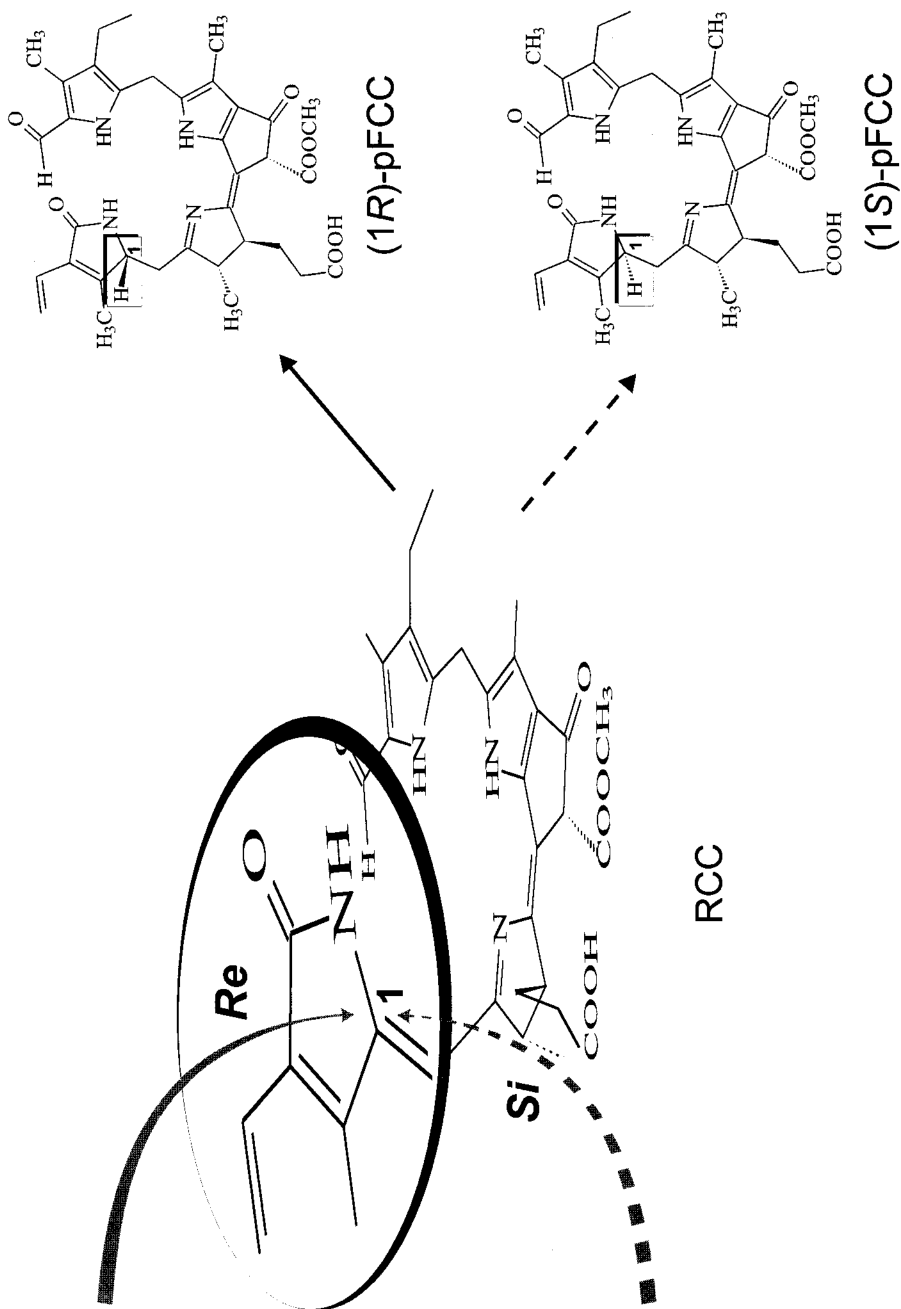

Fig. 4. *Reduction of the red chlorophyll catabolite* (RCC) *by RCC reductase* (RCCR) *introducing a new chiral center at C(1) of the primary FCCs, with the absolute configuration* ((*R*) or (*S*)) *depending upon the type of the reductase*

RCCRs appear to represent a phylogenetically early addition to the chlorophyll catabolic pathway. The development of two types of RCCRs that catalyze a reduction with differing stereochemical outcome, as revealed here, is notable. However, this diversity may point to a functional irrelevance of the stereochemistry of RCC reduction. It is compatible with the view that a major goal of chlorophyll breakdown merely concerns the 'detoxification' of the green plant pigment which may be destructive otherwise (as a photosensitizer) to the regulated processes that occur during senescence [1].

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Experimental Part

1. *General*. Pheophorbide a and ferredoxin were isolated as described in [24]. UV/VIS: *Hitachi-U-3000* spectrophotometer; λ_{max} in nm (rel. ε). CD Spectra: *Jasco-J-715* spectropolarimeter; λ_{max} and λ_{min} in nm (rel. $\Delta\varepsilon$). ^{1}H-NMR: *Varian-Unity plus 500*; δ in ppm with $\delta(CHD_2OD)$ 3.39; J(H,H) in Hz. FAB-MS: *MAT-95* double focusing sector field instrument with fast-atom-bombardment (FAB) positive-ion mode, (Cs ions at 20 keV, 2 µA); matrix glycerine, *m*/*z* (rel. int. in %).

2. *Synthesis and Isolation of* (1ξ,13^{2}R*,17*S*,18*S*)-3^1,3^2-Didehydro-1,4,5,10,17,18,20,22-octahydro-13^2-(methoxycarbonyl)-4,5-dioxo-4,5-secophytoporphyrin* (Ca-FCC-2; 1-*epi*-pFCC; **1**): Ripe red-pepper fruits (*Capsicum annuum* L. cv. Bell Beauty) were obtained from the local store in Zürich. Intact chromoplasts were isolated from 320 g of pericarp tissue by sucrose density centrifugation as described in [15]. Gradient fractions containing chromoplasts were centrifuged (5 min/14000 · *g*) and the plastids, after resuspension in 50 mM *Tris* · HCl (pH 7.6; 16 ml), solubilized for 15 min on ice in the presence of 1% (*v*/*v*) *Triton X-100*. For the synthesis of *Ca*-FCC-2, the mixture was diluted with ferrodoxin (370 µl, 10 U/µl), 50 mM NADPH (320 µl), 50 mM glucose 6-phosphate (320 µl), glucose 6-phosphate dehydrogenase (320 µl, 10 mU/µl), and pheophorbide a (2.6 mg). After 90 min at r.t. in the dark, MeOH (32 ml) was added. The mixture was centrifuged (5 min/14000 · *g*) and the supernatant diluted with H_2O (11.5 ml). It was separated by HPLC (8 × 125 mm column, *Spherisorb ODS-2*, 5 µm; MeOH/100 mM K-phosphate buffer (pH 7.0) 5.5 : 4.5 (*v*/*v*), flow rate 2.4 ml/min; UV detection at 320 nm; fluorescence detection: emission at 450 nm, excitation at 320 nm). The fractions with a retention time of *ca.* 14 min were collected and frozen in liquid N_2. The combined fractions were diluted with H_2O to 10% (*v*/*v*) MeOH and loaded onto a *Waters-C18-SepPak* cartridge. A yellow fraction was eluted with MeOH/40 mM K-phosphate buffer (pH 7.0) 9 : 1 (*v*/*v*) and purified a second time by HPLC as described above. After application to a *C18* cartridge and washing with H_2O (40 ml), the product was eluted with MeOH/H_2O 9 : 1 and the eluate immediately lyophylized at $T < 0°$: *ca.* 0.3 mg of **1** (*Ca*-FCC-2). UV/VIS (H_2O, *ca.* 50 µM): 221 (1.0), 318 (0.7), 358 (0.5). CD (H_2O, *ca.* 50 µM): 226 (– 3.4), 260 (1.0), 330 (2.3), 365 (2.0). NMR Data (CD_3OD, – 20°): *Table 1*. FAB-MS (positive-ion mode): 631.5 (15), 630.5 (50), 629.5 (100, $[M+1]^+$; exact mass determination; 629.2960 ± 0.005), 509.5 (8), 508.5 (37, $[M+1-C_7H_7NO]^+$).

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