Chlorophyll Breakdown – On a Nonfluorescent Chlorophyll Catabolite from Spinach

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Dedicated to Prof. *André M. Braun* on the occasion of his 60th birthday

In extracts of senescent leaves of spinach (*Spinacia oleracea*) that had degreened naturally after the onset of flowering, four colorless compounds, which had characteristic UV/VIS properties of nonfluorescent chlorophyll catabolites (NCCs), were detected by HPLC. From the extracts of 58.7 g of senescent leaves of *Sp. oleracea*, a two-stage HPLC purification procedure provided *ca.* 15 μmol of *So*-NCC-2, the most abundant polar NCC in the leaves of this vegetable. *So*-NCC-2 was isolated as a slightly yellow powder and analyzed by spectroscopic means. The high-resolution mass spectra indicated that *So*-NCC-2 has the same molecular formula as *Hv*-NCC-1 from barley (*Hordeum vulgare*), the first non-green chlorophyll catabolite from a higher plant to be structurally analyzed. Homo- and hetero-nuclear NMR spectroscopy indicated *So*-NCC-2 to have the same constitution as its epimer *Hv*-NCC-1, and to differ from the latter by the configuration at C(1). The catabolite from spinach could be identified with one of the products from OsO_4 dihydroxylation at the vinyl group of the main NCC from *Cercidiphyllum japonicum*. Chlorophyll breakdown in spinach and in *C. japonicum* apparently involves an enzyme-catalyzed reduction that occurs with the same stereochemical sense at C(1), but opposite to that in barley.

1. Introduction. – Chlorophyll breakdown in degreening plants has always been a phenomenon of general interest (see, *e.g.*, [1][2]). According to recent estimates, more than 1000 million tons of chlorophyll are degraded annually on earth (see [2]). In spite of its visibility and its obvious economic and ecological importance, chlorophyll catabolism has remained enigmatic until recently. This remarkable lack of knowledge prevailed for a long time, since the search for catabolic remains of the porphyrinoid moiety of the chlorophylls was futile [3].

Only in the last decade have de-greened products of chlorophyll breakdown from vascular plants been discovered [3][4][5][2]). The first of these to be structurally characterized was *Hv*-NCC-1 (**1**; see *Fig. 1*), a polar 'nonfluorescent' chlorophyll catabolite (NCC) from senescent primary leaves of barley (*Hordeum vulgare*) [4]. The constitution of **1** showed it to be structurally related to chlorophyll a (**2a**) and gave first clues on the major transformations responsible for the color changes that occur in chlorophyll breakdown in green plants. This analysis confirmed an oxygenolytic opening of the porphyrinoid macrocycle to be crucial [4], but revealed a position at the

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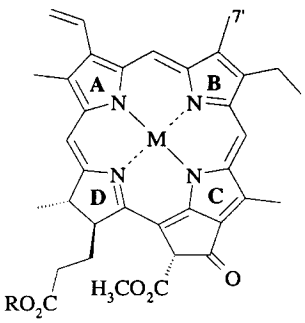
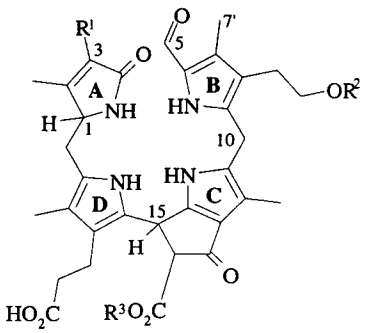
[2]) An interesting parallel development concerns the finding of (presumed) chlorophyll catabolites from other photosynthetic organisms, most notably green algae [6a–e], as well as from sea plankton [6f,g].



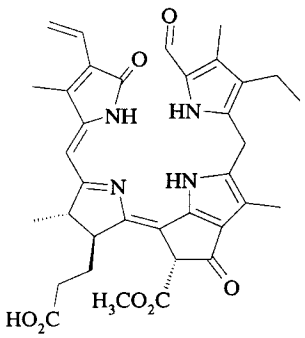


Chlorophylls:

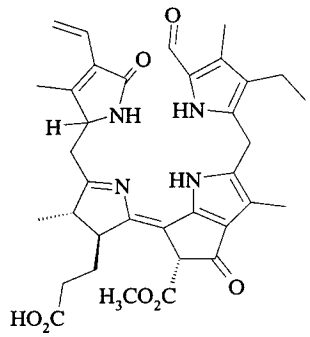
2a chlorophyll a (R = phytol, M = Mg)
2b chlorophyll b (R = phytol, M = Mg)
(7' : formyl instead of methyl)
5 pheophorbide a (R = H, M = 2H)

Nonfluorescent chlorophyll catabolites (NCCs):

1,8 (R^1 = CH(OH)–CH_2OH, R^2 = H, R^3 = CH_3)
3a (R^1 = vinyl, R^2 = malonyl, R^3 = H)
3b (R^1 = vinyl, R^2 = β-glucopyranosyl, R^3 = H)
3c (R^1 = vinyl, R^2 = H, R^3 = H)
4 (R^1 = vinyl, R^2 = H, R^3 = CH_3)



red chlorophyll catabolite
6 (RCC)



'primary' fluorescent chlorophyll catabolite
7 (pFCC)

Fig. 1. *Structural formulae of chlorophylls a and b* (**2a** and **2b**, resp.), *pheophorbide a* (**5**), *and the red chlorophyll catabolite* (**6**, RCC); *constitutional formulae of the 'primary' fluorescent chlorophyll catabolite* (**7**, pFCC) *and the nonfluorescent chlorophyll catabolites* Hv-*NCC-1* (**1**, from barley, *Hordeum vulgare*), *Bn*-NCC-1 (**3a**), *Bn*-NCC-2 (**3b**), Bn-*NCC-3* (**3c**, all from oilseed rape, *Brassica napus*), Cj-*NCC* (**4**, from *Cercidiphyllum japonicum*), *and of* So-*NCC-2* (**8**, from spinach, *Spinacia oleracea*).

macrocycle, which contradicted all earlier proposals concerning chlorophyll breakdown based on chemical-model studies [2].

In the meantime, half a dozen NCCs from vascular plants have been analyzed structurally, such as the *Bn*-NCCs, **3a**–**3c** from oilseed rape (*Brassica napus*) [7] and NCC's (with a common constitution) from *Cercidiphyllum japonicum* (*Cj*-NCC, **4**) [8] and from sweet gum (*Liquidambar styraciflua*) [9] (see *Fig. 1*). All NCCs have shown

the same basic structural pattern, as discovered with **1**. The lack of NCCs, which carry a CHO group at C(7), *i.e.*, which are more closely related to chlorophyll b (**2b**), surprising at first, can be explained meanwhile by the existence of reductases that reduce chlorophyll(ide) b to chlorophyll(ide) a, prior to further breakdown [5b][10][11]. All available data indicate the key step in chlorophyll breakdown in higher plants to be a specific oxygenolytic opening of the porphyrinoid macro-ring of pheophorbide a (**5**), catalyzed by pheophorbide a monooxygenase (PaO)[12][13], the expression of which is senescence specific [5][14].

The product from the action of PaO on **5** is an enzyme-bound form of the red chlorophyll catabolite (RCC, **6**) [15], which is not directly released, but which is directly reduced to primary fluorescent chlorophyll catabolites (pFCCs, **7**) by RCC reductase [5b][16][17]. The reduction step catalyzed by the RCC reductase introduces a new chiral center at C(1) of pFCC, the absolute configuration of which appears to be stereochemically uniform in a given plant [18]. The pFCCs from oilseed rape and from sweet (red) pepper have been structurally characterized and were found to be epimeric at C(1) [16][19]. Consistent with this, two major classes of RCC reductases are indicated to exist in the plant kingdom [18], which were suggested to produce pFCCs that differ by their configuration at C(1) [18][19][3]).

The primary FCCs are detectable in minute quantities only [21] and are rapidly catabolized further to the colorless NCCs [5][22]. The crucial transformation of the chromophore from the slightly yellow FCCs to the colorless NCCs formally involves tautomerization steps that may come about nonenzymatically, *e.g.*, in the slightly acidic environment of the vacuoles [5][23]. Indeed, it was the NCC *Hv*-NCC-1 (**1**) that could be discovered as the first nongreen chlorophyll catabolite from higher plants [4][5], followed, in the meantime, by a set of similar NCCs from other plants [7–9]. The tetrapyrrolic NCCs are deposited into the vacuoles of the senescent leaf [23][24] and have been suggested to represent the final stages of senescence based chlorophyll catabolism in higher plants [5][22]. This question may have to be re-addressed, however, upon the recent characterization of an urobilinogenic chlorophyll catabolite, obtained from isolates from senescent primary leaves of barley and suggested to be the result of an oxidative loss of the CHO group of **1** [25].

Here, we report on the detection and isolation of *So*-NCC-2 (**8**) as the major polar NCC from naturally degreened leaves of the vegetable spinach (*Spinacia oleracea*) and its identification as a stereoisomer of *Hv*-NCC-1 (**1**). In addition, we describe a chemical dihydroxylation of *Cj*-NCC (**4**), the main NCC from *Cercidiphyllum japonicum* [8]. This refunctionalization of **4** gave a mixture of two stereoisomeric tetrapyrroles, one of which turned out to be identical with *So*-NCC-2 (**8**), but not with *Hv*-NCC-1 (**1**). In this way, a stereochemical correlation between *Cj*-NCC (**4**), *So*-NCC-2 (**8**), and *Hv*-NCC-1 (**1**) could be achieved.

Results and Discussion. – Based on the characteristic UV-absorbance properties of NCCs [4][7–9] and analytical HPLC, four polar NCCs could be tentatively identified

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[3]) As had consistently been used earlier [1][2], the atoms in the tetrapyrroles are specified according to the IUPAC numbering of the chlorophylls (see, *e.g.*, [20a], for a recent book on the chemistry (and nomenclature) of linear tetrapyrroles, see [20b]).

in senescent leaves of flowering spinach plants (*Spinacia oleracea*) [26]. The present investigations concern the isolation and structural analysis of the most abundant of these, of *So*-NCC-2 (**8**): from 58.7 g (wet weight) of senescent leaves of flowering spinach plants, *ca.* 10.1 mg (*ca.* 14.9 μmol) of the polar colorless chlorophyll catabolite **8** could be isolated by a multistage purification procedure based on HPLC (see *Exper. Part*)[4]). The catabolite **8** was obtained as a *ca.* 1:10 mixture of two fractions, which equilibrated slowly in neutral aqueous solution (see below). The constitution of *So*-NCC-2 (**8**) was elucidated by spectroscopic means.

The molecular formula of *So*-NCC-2 (**8**) was determined as $C_{35}H_{42}N_4O_{10}$ by high-resolution FAB mass spectrometry in the (+)-ion mode (experimental base peak $[M+H]^+$ at *m/z* 679.299, potassium complex $[M+K]^+$ at *m/z* 717.4). The same molecular formula was previously derived from the mass spectrum of *Hv*-NCC-1 (**1**). In the spectra of both **1** and **8**, loss of 157 mass units (due to ring A) was observed as a characteristic fragmentation mode [4][28].

The UV/VIS spectrum of **8** matched that of **1**, and, in both cases, the absorbance maximum at longest wavelength appeared at 315 nm, compatible with an 2-formylpyrrole moiety. The CD spectra of both catabolites were nearly congruent, also suggesting that the stereogenic centres C(15) and C(13^3) have the same configurations in **1** and **8** (see below).

The constitution of *So*-NCC-2 (**8**) was determined unambiguously by (homo- and heteronuclear) NMR experiments. The signals of 33 of the 42 protons were detected in 500-MHz ^{1}H-NMR spectra of **8** in D_2O (or in CD_3OD): one *singlet* for the CHO proton, five *singlets* for the Me groups, one of which was indicated by its chemical shift to be a part of a methyl ester function. No unsubstituted peripheral ethyl or vinyl groups were detected. Seven of the ten O-atoms were accommodated by one methyl ester, one COO, one lactam, and two CO groups. The remaining three O-atoms were assigned according to the typical downfield shift of the H- and C-atoms CH(3^1), CH_2(3^2), and CH_2(8^2) to OH groups at these peripheral substituents. The nine exchanging protons were assigned to four NH, COOH, three OH groups, and the H−C(α) of a β-keto ester moiety. The connectivity between the 35 C-atoms, as well as that of the C-bound H-atoms could be derived from ROESY, HSQC, TOCSY, and HMBC spectra (see, *e.g.* [29]), from which signals of all 35 C-centres could be detected and assigned (see *Fig. 2*). According to this analysis, the constitution of *So*-NCC-2 (**8**) was determined to be identical with that of *Hv*-NCC-1 (**1**).

Analysis of samples of *So*-NCC-2 (**8**) and *Hv*-NCC-1 (**1**) by HPLC revealed different retention times (see *Fig. 3*), complementing the finding of small, but significant, differences in the NMR data sets of the two NCCs. Accordingly, the two catabolites, **1** and **8**, were indicated to be nonidentical and thus to be stereoisomers.

Determination of the configurational relationship between **1** and **8**, based on (and verifying) the identity of their constitution: of the four stereogenic centers in **1** and **8** (C(1), C(3^1), C(13^2), and C(15)), the latter two stereogenic centers were indicated to have the same relative configuration in **1** and **8** according to ROESY spectra, *i.e.*, the MeOOC group and ring D were found to be in the thermodynamically presumably

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[4]) This amount can be estimated to correspond to *ca.* 20% of the total chlorophyll content of green spinach leaves [27].

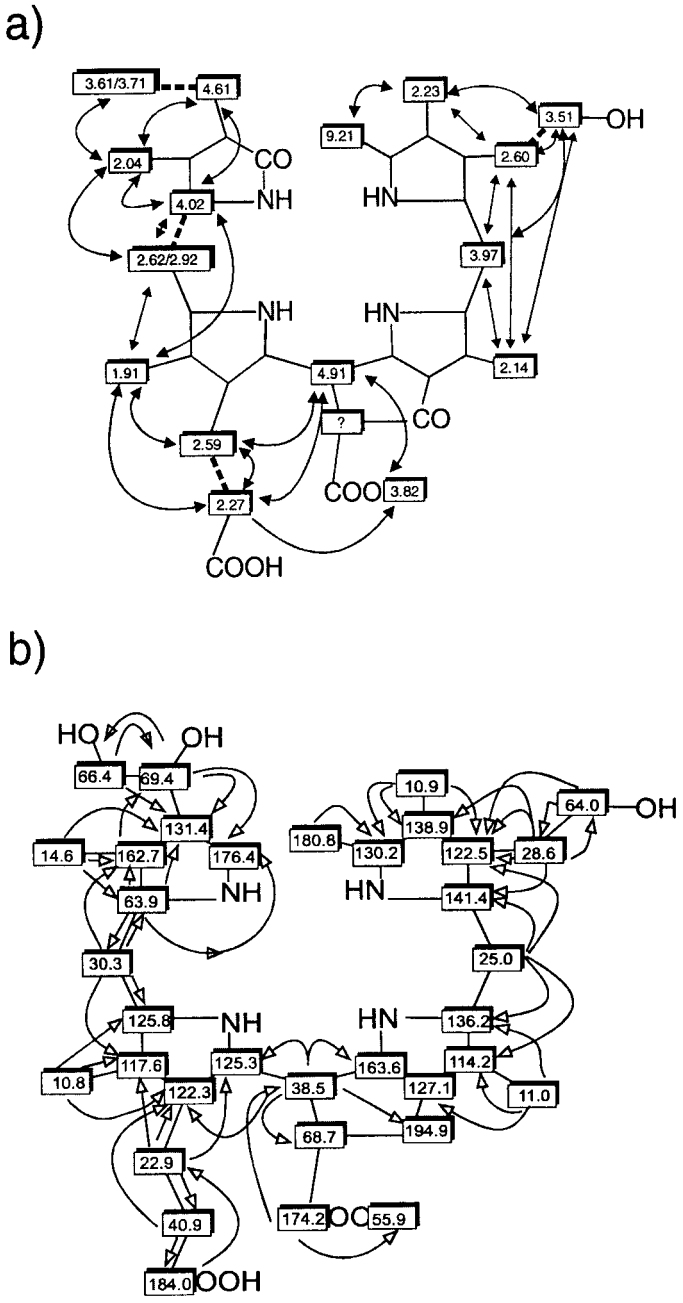

Fig. 2. a) *Assignment of the* ^{1}*H-NMR signals of* **8** *in* D_2O (26°), *NOE correlations* (arrows) *from a ROESY spectrum and* J*(H,H) correlations* (dashed lines) *from TOCSY.* b) *Assignment of the* ^{13}*C-NMR signals of* **8** *in* D_2O (26°) *from an* $^{1}H,^{13}C$*-HSQC spectrum and* ^{2}J*(C,H)/*^{3}J*(C,H) correlations from an* $^{1}H,^{13}C$*-HMBC spectrum* (arrows pointing from ^{1}H site to correlated ^{13}C).

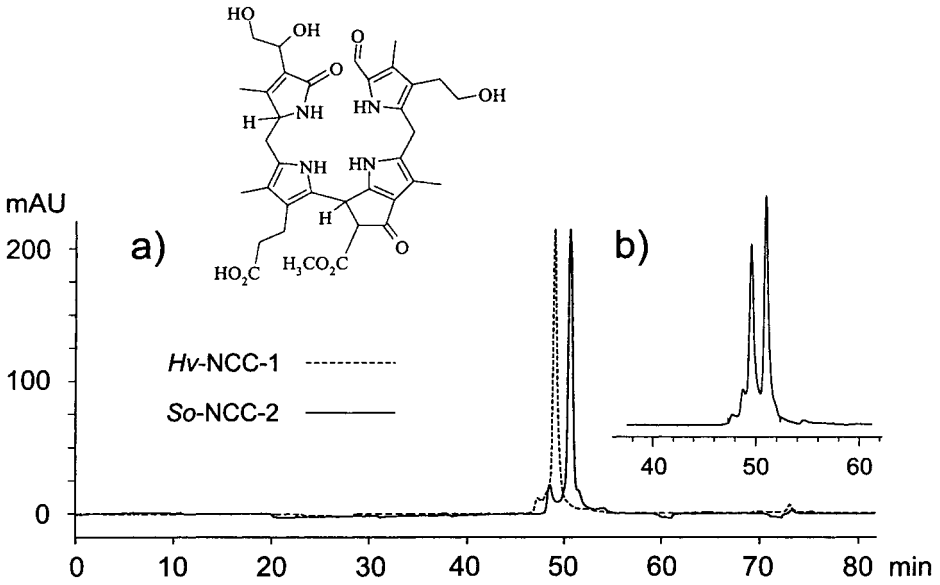

Fig. 3. *Analytical HPLC indicates* Hv-*NCC-1* (**1**) *and* So-*NCC-2* (**8**) *to be stereoisomers* (common constitutional formula of **1** and **8** is shown). a) *Analysis of* **1** *and* **8** *injected separately*; b) *analysis of co-injected* **1** *and* **8** (*a* and *b:* flow rate 500 µl/min; eluents: $A = 100$ mM K_3PO_4 pH 7.0, $B =$ MeOH; 0–10 min: A/B 80:20 (*v*/*v*), 10–70 min: linear gradient A/B 80:20 to 40:60 (*v*/*v*); 70–80 min: A/B 40:60 (*v*/*v*); detection at 320 nm).

more favorable mutual *trans*-arrangement. Accordingly, the questions concerning the stereoisomeric nature of **1** and **8** could be narrowed down further, considering the possible result of a nonstereoselective dihydroxylation of *Cj*-NCC (**4**) at the vinyl group, which would produce NCCs with the same constitution as **1** or **8**. An encouraging exploratory dihydroxylation of **10**[5]), the methyl ester of *Cj*-NCC **4**, with OsO_4 confirmed the expected tendency of the vinyl group of **10** to undergo regioselective but stereo-unselective oxidation and a 1:1 mixture of the C(3^1)-epimeric dihydroxylation products **11a** and **11b** (corresponding to *So*-NCC-2 methyl esters) could be obtained in nearly quantitative yield (at *ca.* 18% conversion; see *Scheme*). The epimeric nature at C(3^1) of **11a**/**11b** could be derived from analysis of the ^{1}H-NMR spectrum, where three Me group *singlets* are clearly split in a ratio of 1:1. Only a modest stereoselectivity was observed also in an exploratory experiment based on the dihydroxylation technique by *Sharpless* and co-workers [30] and with *O*-(4-chlorobenzoyl)dihydroquinine as chiral additive.

Stereo-unselective osmium tetroxide oxidation of *Cj*-NCC (**4**) was thus considered to give **1** or **8**, or a C(15)-epimer of either one of these NCCs (see *Scheme*). In the event, OsO_4 oxidized **4** to a mixture of dihydroxylation products, which were separable by HPLC into a minor more-polar fraction **9a′**/**9b′** and a less-polar major fraction **9a**/**9b**.

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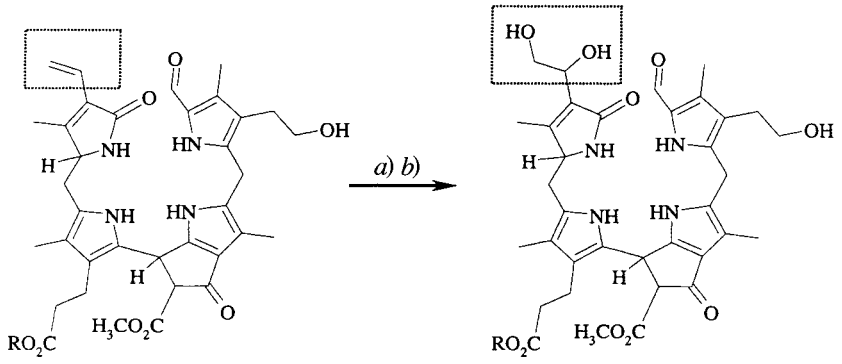
[5]) *Cj*-NCC (**4**) was esterified under conditions reported earlier for the NCC from sweet gum (*Liquidamber styraciflua*), which has the same constitution as **4**, but the structure of which was not compared experimentally with that of **4** [9]; use of different solvent systems for the NMR spectra so far precludes a meaningful spectral comparison.

Scheme. *OsO_4 Oxidation of* i) Cj-*NCC* (**4**) *produces* So-*NCC-2* (**8**), *roughly in a* 1:1 *mixture with its C(3¹)-epimer, and* ii) *of* Cj-*NCC methyl ester* **10** *gives the corresponding mixture of epimers* **11a/11b**



4 (*Cj*-NCC): R = H
10 R = Me

9a/9b: R = H
11 R = Me

a) OsO_4, 0.5% in THF, $-15°$ to $-5°$; b: 0.125M $NaHSO_3$ in H_2O/MeOH 1:1, 0°).

The two fractions slowly interconverted in solution to give a *ca.* 1:10 mixture at equilibrium and were obtained in a combined yield of *ca.* 65%[6]). The major dihydroxylation fraction **9a/9b** had HPLC characteristics identical to those of *So*-NCC-2 (**8**) (see *Fig. 4*), but clearly different from those of *Hv*-NCC-1 (**1**). The UV and CD spectra of the dihydroxylation fraction **9a/9b** were also virtually indistinguishable from those of **8**, but analysis by ^{1}H-NMR spectra indicated splitting of some of the Me-group signals. These data are consistent with a *ca.* 1:1 mixture of two compounds, as expected for a diastereo-unselective dihydroxylation. In the ^{1}H-NMR spectra of the fraction **9a/9b** in D_2O solution, only the signals of the Me group at C(2) and of the 1,2-dihydroxyethyl group at C(3) showed any noticeable splitting, in support of the existence of two epimers at C(3¹). In the two solvent systems investigated (D_2O and CD_3OD), a further significant difference between the ^{1}H-NMR spectra of the fraction **9a/9b** and of *So*-NCC-2 (**8**) was not discernible. Likewise, the 32 detectable ^{13}C-NMR signals (in the HSQC and HMBC spectra in D_2O) of the fraction **9a/9b** did not show any significantly different chemical-shift values compared to those in the spectrum of *So*-NCC-2 (**8**).

This study thus identifies *So*-NCC-2 (**8**) with a product of dihydroxylation of *Cj*-NCC (**4**), establishing a stereochemical (and confirming the spectroscopically deduced constitutional) correlation between these two NCCs. This correlation indicates *So*-NCC-2 (**8**) from *Sp. oleracea* and *Cj*-NCC (**4**) from *C. japonicum* (and the other NCCs from spinach and *C. japonicum*) to have the same absolute configuration at their C(1), as the result of the presumed common stereoselectivity of the corresponding RCC reductases (RCCR). The reductase in *C. japonicum* would, therefore, be

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[6]) From HPLC analysis of mixtures, equilibrated after 24 h in MeCN/100 mM aqueous K_3PO_4 pH 7/H_2O 2:3:5 (*v*/*v*), and starting with nearly uniform fractions **9a/9b** or **9a′/9b′**.

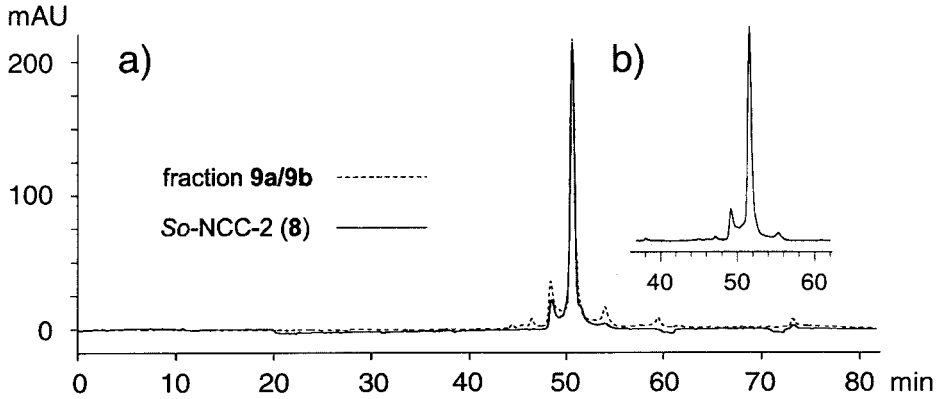

Fig. 4. *Comparison of* So-*NCC-2* (**8**) *and of fraction* **9a**/**9b** *from dihydroxylation of* Cj-*NCC* (**4**) *by analytical HPLC.* a) *Analysis of* **9a/9b** *and* **8** *injected separately*; b) *analysis of co-injected* **9a/9b** *and* **8** (*a* and *b:* flow rate 500 µl/min; eluents: $A = 100$ mM K_3PO_4 pH 7.0, $B =$ MeOH; 0–10 min: A/B 80:20 (*v*/*v*); 10–70 min: linear gradient A/B 80:20 to 40:60 (*v*/*v*); 70–80 min: A/B 40:60 (*v*/*v*); detection at 320 nm).

suggested to be of the RCCR-2 class, as in spinach and in red pepper [18][19] (and in contrast to the reductases of barley and oilseed rape, which are of the RCCR-1 class [16][19]). The identification of one of the dihydroxylation products **9a**/**9b** with **8** also implies the same absolute configuration of C(15) in **4** and **8**. This conclusion is supported by the similarity of the long wavelength part of the CD spectra of **1** and **8** (see above). On the same basis, C(15) was tentatively assigned the same configuration in **1** and **8** also. These assignments would indicate the hypothetical (enzymatic or nonenzymatic) tautomerization that furnishes the NCCs from the FCCs to attach the H-atom to C(15) with a common (diastereofacial) selectivity and independent of the configuration at C(1) in the three cases of senescent leaves of barley, of *C. japonicum*, and of spinach. The preferred relative configuration at C(15) and C(13^2), with H-C(15) *cis* to the MeOOC group at C(13^2) in **1** and **8** (or a COOH group in **3a**) [4][7], appears to be a broad and possibly common characteristic of the isolated NCCs. This structural aspect is likely to be the result of nonenzymatic equilibration reactions at the acidic C(13^2) position, which adjust its configuration to that at C(15). The latter process would be assumed to occur in the senescent plant leaf, but also takes place in the isolated NCCs. The present study, therefore, suggests *Hv*-NCC-1 (**1**) and *So*-NCC-2 (**8**) to have the same absolute configuration at C(15) (and at C(13^2)), but to differ from each other in the configuration at C(1)[7]).

Conclusion. – This work concerns the preliminary identification of nonfluorescent chlorophyll catabolites (NCCs) from senescent leaves of a vegetable, of spinach, and the structural characterization of the most abundant of these, of *So*-NCC-2 (**8**). The analysis of **8** as a stereoisomer of the *Hv*-NCC-1 (**1**) [4] underlines the relevance of extensive peripheral hydroxylations in the course of chlorophyll breakdown in higher plants under natural growth conditions. The same functional groups were discovered in

[7]) In addition, the NCC's **1** and **8** possibly also differ by their configuration at C(3^1)

1, which was obtained from primary leaves of barley that were degreened in artificial darkness. The nonidentity of **1** and **8**, and their epimeric nature at C(1) are consistent with the occurrence of two classes of RCC reductases [16][18][19] in these higher plants. The stereochemical diversity of the RCC reductases may not be untypical of detoxifying enzymatic processes and has been suggested to point to the lack of physiological relevance of the configuration at C(1) in the FCCs and their further catabolic products [19].

The structural basis for the epimeric nature of **1** and **8** was elucidated with the help of a stereo-unselective dihydroxylation of **4**, which provided similar amounts of the isomers at C(3^1). Whereas the constitutions of NCCs can be delineated with little ambiguity, the determination of the relative configuration of the (up to four) stereocenters in the NCCs remains a problem, and the information on the absolute configuration is still elusive. Here, we have provided an example, where natural NCCs from three different plant sources could be correlated stereochemically. By this means, the type of RCC reductase [19] involved in one of these plants (*C. japonicum*) could also be deduced indirectly.

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Experimental Part

General. Reagents used were reagent-grade commercials, and solvents were distilled before use. Reagents and HPLC solvents were from *Fluka* (Buchs, Switzerland) and from *Merck* (Darmstadt, Germany). TLC: analytical (0.25-mm silica gel *60 F_{254}*) and prep. plates (20 cm × 20 cm, 1 mm silica gel *60 F_{254}*) were from *Merck*, Darmstadt, Germany. *Sep-Pak-C18* cartridges were from *Waters Associates.* Anal. HPLC: column: *Hypersil ODS* 5 μm, 240 × 8.4 mm o.d or 250 × 4.6 mm i.d., pump: *Gynkotek M480G* with vacuum on-line degasser, *HP 1100* QuatPump. Prep. HPLC: column: *Hypersil ODS* 5 μm, 320 × 22 mm o.d. or 250 × 21.2 mm i.d.; pump: *Gynkotek M300*, solvents were degassed by sonication, *HP 1100 QuatPump*; detectors: *Gynkotek* diode-array detector *UVD 340*, *HP 1100* diode-array detector, *Hitachi SPD-6AV* UV/VIS detector. All chromatograms were taken at r.t., and data were processed by *Gynkotek* HPLC-data system *Gynkosoft 5.50* or *HP Chemstation* for 3D. UV/VIS-Spectra: *Hitachi U3000* spectrophotometer; λ_{max}(nm)/(log ε). CD Spectra: *Jasco J715* spectropolarimeter; λ_{max}(nm) and λ_{min}(nm)/($\Delta\varepsilon$). NMR Spectra: *Varian Unity$_{plus}$ 500* or *Bruker AM-300* ($\delta(C^1HCl_3) = 7.26$ ppm and $\delta(^{13}CDCl_3) = 77.0$ ppm, $\delta(C^1HD_2OD) = 3.31$ ppm and $\delta(^{13}CD_3OD) = 49.0$ ppm, $\delta(HDO) = 4.79$ ppm) [31]). MS: *Finnigan-MAT 95-S* pos.-ion mode; FAB-MS: Cs gun, 20 keV, matrix: 3-nitrobenzyl alcohol (NOBA); ESI-MS: 3.2 kV, solvents: H_2O/MeOH mixtures.

Isolation of So-NCC-2 (**8**) *from Spinach*. Spinach plants (*Spinacia oleracea*) were grown in the green house. After the onset of flowering, senescent leaves (58.7 g) were collected, washed with distilled H_2O, and frozen in liq. N_2. An extract containing NCCs was obtained by mixing and suspending the ground leaf tissue once in 120 ml and three times with 60 ml of 20 mM K_3PO_4 buffer (pH 7.0)/MeOH 1:1 *v*/*v*, followed each time by centrifugation (5 min at 10000 × g) and separation of the supernatants. The combined supernatants were diluted with 300 ml of MeOH and centrifuged once more. The resulting supernatant was then concentrated under reduced pressure, lyophilized, and dissolved in 20 ml H_2O. By prep. HPLC (*Hypersil ODS*, MeOH/K_3PO_4 buffer, pH 7.0, with a linear gradient (20–60% MeOH, in 70 min); flow rate 5 ml/min; UV detection at 320 nm) fractions containing *So*-NCC-2 (**8**) were obtained (t_R 61 min). The fractions with **8** were diluted 1:1 with H_2O (*ca.* 25 ml), applied to a *Waters Sep-Pak-C18* cartridge, which was washed with H_2O to remove the buffer, and with MeOH to elute a slightly yellow fraction of **8**. The solvents of the collected eluates with **8** were removed *in vacuo*, and the residue was dissolved in *ca.* 3.5 ml of HPLC eluent (MeOH/0.1M K_3PO_4-phosphate, pH 7.0, 2:3 *v*/*v*) and purified by a second run of prep. (isocratic) HPLC (as described above), but with MeOH/0.1M K_3PO_4 buffer, pH 7.0, 2:3 (*v*/*v*) and a flow rate of 5 ml/min. The *So*-NCC-2 fraction (t_R 30–34 min) was collected, the

solvent volume was reduced from 50 to *ca.* 30 ml in high vacuum (below r.t.), and the residual soln. was applied to a *Sep-Pak-C18* cartridge. After washing with H_2O (20 ml), *So*-NCC-2 (**8**) was eluted with MeOH, and the eluate was dried (at high vacuum) at $T < 0°$ to yield 10.1 mg (14.9 μmol) of *So*-NCC-2 (**8**).

Data of So-*NCC-2* (= *1,4,5,10,15,20-Hexahydro-3¹,3²,8²-trihydroxy-13²-(methoxycarbonyl)-4,5-dioxo-22H,24H-4,5-secophytoporphyrin*; **8**): UV/VIS (0.02M K_3PO_4 buffer pH 7.0, $c = 7.9 \times 10^{-5}$ M): λ_{max}(nm)/(log ε) = 207 (4.14), 243 (3.86), 315 (3.93). CD (in 0.02M K_3PO_4 buffer pH 7.0, $c = 7.9 \times 10^{-5}$ M): $\lambda_{min/max}$(nm)/($\Delta\varepsilon$) = 204 (−6.30), 226 (9.27), 258 (−2.16), 250 (−2.25), 282 (−7.27), 322 (1.99). NMR: *a*) In CD_3OD: ^{1}H-NMR (500 MHz, 26°): 1.94, 2.02, 2.08, 2.26 (4*s*, Me(2¹), Me(7¹), Me(12¹), Me(18¹)); 2.32 (*m*, CH_2(17²)); 2.53 (*dd*, *J* = 8.2, 14.4, H_A−C(20)); 2.62 (*m*, H_A−C(17¹)), superimposed by 2.63 (*t*, *J* = 6.0, CH_2(8¹)); 2.74 (*m*, H_B−C(17¹)); 2.87 (*dd*, *J* = 3.7, 14.2, H_B−C(20)); 3.47 (*m*, CH_2(8²)); 3.63 (*m*, H_A−C(3²)); 3.68 (*m*, H_B−C(3²); 3.74 (*s*, Me(13⁵)); 3.97 (br. *s*, CH_2(10)); superimposed by 3.99 (*m*, H−C(1)); 4.56 (*m*, H−C(3¹)); 4.89 (*s*, H−C(15)); 9.39 (*s*, H−C(5)). ^{13}C-NMR (125 MHz, 26°): 8.8 (C(7¹)); 9.0 (C(12¹)); 9.3 (C(18¹)); 12.4 (C(2¹)); 21.8 (C(17¹)); 23.6 (C(10)); 27.9 (C(8¹)); 29.5 (C(20)); 37.1 (C(15)); 39.3 (C(17²)); 53.1 (C(13⁵)); 62.6 (C(8²)); 62.7 (C(1)); 65.8 (C(3²)); 68.0 (C(13²)); 68.6 (C(3¹)); 112.4 (C(12)); 115.3 (C(18)); 120.8 (C(17)); 121.1 (C(8)); 124.6 (C(16)); 124.6 (C(19)); 126.1 (C(13)); 129.5 (C(6)); 131.7 (C(3)); 134.5 (C(11)); 135.5 (C(7)); 139.3 (C(9)); 159.1 (C(2)); 161.3 (C(14)); 172.1 (C(13³)); 175.1 (C(4)); 177.6 (C(5)); 181.3 (C(17³)). *b*) In D_2O: ^{1}H-NMR (500 MHz, 26°): 1.91, 2.04, 2.14, 2.23 (4*s*, Me(2¹), Me(7¹), Me(12¹), Me(18¹)); 2.27 (*m*, CH_2(17²)); 2.59–2.66 (*m*, CH_2(17¹), CH_2(8¹), H_A−C(20)); 2.92 (*dd*, *J* = 3.9, 13.7 H_B−C(20)); 3.51 (*t*, *J* = 6.4, CH_2(8²)); 3.61 (*dd*, *J* = 5.8, 11.7, H_A−C(3²)); 3.71 (*dd*, *J* = 6.8, 11.7, H_B−C(3²)); 3.82 (*s*, Me(13⁵)); 3.98–4.02 (*AB*, J_{AB} = 17.7, CH_2(10)); 4.04 (br., H−C(1)); 4.61 (*dd*, *J* = 6.8, 5.8, H−C(3¹)); 4.91 (*s*, H−C(15)); 9.21 (*s*, H−C(5)). ^{13}C-NMR (75 MHz, from HSQC and HMBC, 25°): 10.8 (C(18¹)); 10.9 (C(7¹)); 11.0 (C(12¹)); 14.6 (C(2¹)); 22.9 (C(17¹)); 25.0 (C(10)); 28.6 (C(8¹)); 30.3 (C(20)); 38.5 (C(15)); 40.9 (C(17²)); 55.9 (C(13⁵)); 63.9 (C(1)); 64.0 (C(8²)); 66.4 (C(3²)); 68.7 (C(13²)); 69.4 (C(3¹)); 114.2 (C(12)); 117.6 (C(18)); 122.3 (C(17)); 122.5 (C(8)); 125.3 (C(16)); 125.8 (C(19)); 127.1 (C(13)); 130.2 (C(6)); 131.4 (C(3)); 136.2 (C(11)); 138.9 (C(7)); 141.4 (C(9)); 162.7 (C(2)); 163.6 (C(14)); 174.2 (C(13³)); 176.4 (C(4)); 180.8 (C(5)); 184.0 (C(17³)); 194.9 (13¹)). FAB-MS (glycerol matrix): 719.4 (20), 718.4 (37), 717.4 (46, $[M+K]^+$), 716.4 (10); 681.5 (23), 680.5 (67), 679.38 (100, $[M+H]^+$), 678.4 (13); 591.4 (20); 523.5 (35), 522.5 (54, [M + 1 − ring A]$^+$). HR-MS: 679.299 ($C_{35}H_{43}N_4O_{10}$; calc. 679.298).

Cj-*NCC* (= *3¹,3²-Didehydro-1,4,5,10,15,20-hexahydro-8²-hydroxy-13²-(methoxycarbonyl)-4,5-dioxo-22H,24H-4,5-secophytoporphyrin;* **4**). The major NCC from senescent leaves of *Cercidiphyllum japonicum* was obtained essentially as described in [8]: the main NCC fraction from 250 g (fresh weight) of senescent leaves was isolated as a slightly yellow powder after chromatography on prep. TLC plates. 84.8 mg of *Cj*-NCC (**4**) were obtained and were identified by comparison of the UV/VIS, ^{1}H-NMR, and FAB-MS data [8].

Epimer Mixture **9a/9b** *from* **4**. A soln. of **4** (7.9 mg, 0.012 mmol) in THF (2 ml, freshly distilled over Na) was cooled under Ar to −15°, and OsO_4 in dry THF (0.5%, 0.61 ml, 0.012 mmol) was added slowly under vigorous stirring. The mixture was allowed to warm to −5°, and stirring was continued. After 4 h, another portion of the OsO_4 soln. (0.5% 0.61 ml, 0.012 mmol) was added, and stirring was continued for another 16 h at −5°. TLC (CH_2Cl_2/MeOH/H_2O 80:18:2 (*v/v*)) showed an increasing amount of a product slightly more polar than the educt. The soln. was warmed to 0°, and a 0.125M $NaHSO_3$ soln. (1.0 ml, 0.125 mmol) in MeOH/H_2O 1:1 (*v/v*) was added. After 10 min, the mixture (sharp new product spot by TLC) was transferred into a 50-ml separating funnel, diluted with K_3PO_4 (15 ml, 100 mM, pH 5) and shaken with CH_2Cl_2 (3 × 10 ml). The org. layers were filtered through a plug of cotton wool. The remaining aq. phase was saturated with NaCl and extracted twice with AcOEt (20 ml). The resulting org. layer was washed with K_3PO_4 (20 ml, 100 mM, pH 5, to remove traces of osmium), dried ($MgSO_4$) and filtered. The org. layers contained the tetrapyrrolic products (according to UV/VIS spectra) and were concentrated under high vacuum for further purification by prep. HPLC (MeCN/K_3PO_4 (100 mM, pH 7)/H_2O 2:3:5 (*v/v*), 5 ml/min. flow, UV detection at 310 nm). Two fractions were collected from the CH_2Cl_2 fraction: fraction **9a′/9b′** at t_R 10–11 min and (the main) fraction **9a/9b** at t_R 11–13 min. The two fractions were applied to *Sep-Pak-C18* cartriges, washed with H_2O (50 ml), and eluted with MeOH (3 ml). The filtrates were concentrated and dried under high vacuum to give 3.0 mg of the major fraction **9a/9b** and 0.4 mg of **9a′/9b′**, which were both submitted to spectroscopic analysis.

Data of **9a/9b**: UV/VIS (MeOH): λ_{max}(rel. ε) = 212 (1.0), 246 (sh, 0.52), 314 (0.58); CD (MeOH): λ_{max}(rel $\Delta\varepsilon$) = 225 (1.0), 252 (sh, −0.14), 282 (−0.77), 315 (+0.14). ^{1}H-NMR (500 MHz, D_2O, 26°): 1.90 (*s*, 3 H); 2.03/2.04 (2*s*, 3 H); 2.15 (*s*, 3 H); 2.23 (*s*, 3 H); 2.25–2.35 (*m*, 2 H); 2.58–2.67 (*m*, 5 H); 2.93 (*m*, 1 H); 3.53 (*m*, 2 H); 3.57–3.63 (*m*, 1 H); 3.68–3.78 (*m*, 2 H); 3.82 (*s*, 3 H); 3.95–4.05 (*m*, 3 H); 4.59 (*m*, 1 H); 4.90 (*s*, 1 H); 9.22 (*s*, 1 H). ^{13}C-NMR (125 MHz, from HSQC and HMBC in D_2O, 26°): 11.1 (C(7¹)); 11.2 (C(18¹)); 11.4 (C(12¹)); 14.9 (C(2¹)); 22.9 (C(17¹)); 25.3 (C(10)); 28.7 (C(8¹)); 30.3 (C(20)); 38.6 (C(15)); 40.4 (C(17²)); 55.9 (C(13⁵)); 63.9 (C(8²)); 64.2 (C(1)); 66.2 (C(3²)); 69.4 (C(3¹)); 114.5 (C(12)); 117.7 (C(18)); 122.1 (C(17)); 122.6 (C(8)); 125.8

(C(16)); 126.2 (C(19)); 127.3 (C(13)); 130.7 (C(6)); 131.7 (C(3)); 136.6 (C(11)); 139.2 (C(7)); 141.7 (C(9)); 162.7 (C(2)); 163.9 (C(14)); 174.3 (C(13^3)); 177.0 (C(4)); 184.0 (C(17^3)). ^{1}H-NMR (500 MHz, CD_3OD, 26°): 1.92, 1.93 (2*s*, 3 H); 2.02 (*s*, 3 H); 2.08, 2.09 (2*s*, 3 H); 2.25, 2.26 (2*s*, 3 H); 2.29–2.75 (*m*, 7 H); 2.86 (*m*, 1 H); 3.42–3.53 (*m*, 2 H); 3.59–3.71 (*m*, 2 H); 3.74 (*s*, 3 H); 3.92–4.07 (*m*, 3 H); 4.55 (*m*, 1 H); 4.87 (*s*, 1 H); 9.36 (*s*, 1 H). FAB-MS: 719.2 (30), 718.1 (32), 717.3 (38, $[M+K]^+$), 703.2 (38), 702.2 (58), 701.2 (64, $[M+Na]^+$), 681.2 (50), 680.2 (85), 679.20 (100, $[M+H]^+$), 678.2 (46), 677.2 (36), 524.4 (44), 523.4 (64), 522.5 (80, $[M-$ring A$]^+$).

The fraction **9a′/9b′** was collected likewise by HPLC (MeCN/100 mM K_3PO_4, pH 7/H_2O 2:3:5 (*v*/*v*)) and was stored at −25°. It was then warmed to r.t., and the samples were analyzed by HPLC (stored at r.t. during 24 h). The remaining part of the fraction **9a′/9b′** was filtered through a *Sep-Pak* cartridge (as described above), dried (0.4 mg), and submitted to spectroscopic analysis. HPLC retention times, UV/VIS, CD (MeOH), and ^{1}H-NMR (CD_3OD) data were identical to those of fraction **9a/9b**.

The extract with AcOMe was worked up in the same way by prep. HPLC (MeCN/K_3PO_4, 100 mM, pH 7/H_2O 15:40:45 (*v*/*v*), 5 ml/min flow). Product fractions with t_R 30–40 min were collected and worked up (as described above for fraction **9a/9b**, yield: 2.0 mg) and identified with **9a/9b** by HPLC, UV/VIS, CD (MeOH), FAB-MS, and ^{1}H-NMR (CD_3OD) data. In addition, an apolar fraction with starting material **4** was isolated by HPLC and worked up likewise (*ca.* 2 mg) and identified with ^{1}H-NMR. Total yield of fractions **9a/9b**: 5.4 mg (65%).

*Methyl 3^1,3^2-Didehydro-1,4,5,10,15,20-hexahydro-8^2-hydroxy-13^2-(methoxycarbonyl)-4,5-dioxo-22*H,*24*H*-4,5-secophytoporphyrinate* (**10**). To a soln. of **4** (84.8 mg, 0.131 mmol) in CH_2Cl_2/MeOH 1:2 (*v*/*v*; 15 ml), cooled to 0°, Et_3N (20.5 mg, 0.157 mmol) and [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphat ('*Castro*'s reagent'; 66.7 mg, 0.157 mmol) were added with rigorous stirring, and the mixture was allowed to warm to r.t. After 4 h, the solvent was evaporated in high vacuum, and the crude product was purified by TLC with $CHCl_3$/MeOH/H_2O 80:15:2 (*v*/*v*). The zone containing **10** ($R_f \approx 0.6$) was scratched from the plate and washed from the silica gel with CH_2Cl_2/MeOH 7:3 (*v*/*v*; 70 ml). The soln. was dried *in vacuo*, and the product **10** was precipitated with hexane and dried under high vacuum. Yield: 67.8 mg (78.5%). UV/VIS (CH_2Cl_2): $\lambda_{max}(\varepsilon)$ 221 (1.0), 241 (0.73), 311 (0.55). ^{1}H-NMR (500 MHz, CD_3OD, 26°): 1.89 (*s*, Me(18^1)); 1.95 (*s*, Me(2^1)); 2.13 (*s*, Me(12^1)); 2.26 (*s*, Me(7^1)); 2.38–2.44 (*m*, H_B–C(20), CH_2(17^2)); 2.55–2.67 (*m*, 3 H, CH_2(8^1), CH_2(17^1)); 2.85 (*dd*, H_A–C(20)), 3.45–3.56 (*m*, CH_2(8^2)); 3.60 (*s*, MeOOC–C(17^2)); 3.75 (*d*, H–C(13^2)); 3.77 (*s*, MeOOC–C(13^2)); 3.93, 3.94 (*AB*$_{gem}$, CH_2(10)); 4.02 (*m*, H–C(1)); 5.35 (*dd*, H_{cis}–C(3^2)); 6.08 (*dd*, H_{trans}–C(3^2)); 6.43 (*dd*, 1 H H–C(3^1)); 9.35 (*s*, H–C(5))[8]). FAB-MS: 661.4 (9), 660.4 (36), 659.4 (92, $[M+H]^+$), 537.3 (35), 536.3 (100, $[M-$ring A $]^+$).

*Methyl 1,4,5,10,15,20-Hexahydro-3^1,3^2,8^2-trihydroxy-13^2-(methoxycarbonyl)-4,5-dioxo-22*H,*24*H*-4,5-seco-phytoporphyrinate* (**11**). A soln. of **10** (8 mg, 0.0124 mmol) in THF (1 ml, freshly distilled over Na) was cooled under Ar to 0°. A 0.5% soln. of OsO_4 (0.6 ml, 3.0 mg, 0.0118 mmol) in dry THF was added slowly under vigorous stirring. TLC ($CHCl_3$/MeOH/H_2O 95:10:1 (*v*/*v*)) showed an increasing amount of a new product, slightly more polar than **10**. After 2 h, a 0.125M $NaHSO_3$ soln. (0.5 ml, 0.125 mmol) in MeOH/H_2O 1:1 (*v*/*v*) was added, and stirring was continued. After 10 min, the product **11** had developed (new sharp spot on TLC), and the mixture was transferred into a 50-ml separating funnel, diluted with H_2O (20 ml), and shaken with CH_2Cl_2 (3 × 10 ml). The org. layers were filtered through a plug of cotton wool and analyzed by UV/VIS spectra. The soln. was concentrated under high vacuum and purified by prep. TLC in $CHCl_3$/MeOH/H_2O 90:10:1 (*v*/*v*). The zone containing **11** (R_f 0.35) was scratched from the plate and washed from the silica gel with CH_2Cl_2/MeOH 7:3 (*v*/*v*; 40 ml). The product **11** was precipitated from a conc. CH_2Cl_2 soln. with hexane and dried under high vacuum. Yield: 1.6 mg (18%, 99% with respect to conversion of **10**). A second fraction (at R_f *ca.* 0.5) containing starting material was recovered from the TLC plates, identified as **10** by ^{1}H-NMR and quantified by UV/VIS spectroscopy (6.4 mg).

Data of **11**: UV/VIS (CH_2Cl_2): λ_{max}(rel. ε) = 220 (1.0), 243 (sh, 0.68), 310 (0.53). ^{1}H-NMR (500 MHz, CD_3OD, 26°): 1.90, 1.91 (2*s*, Me(18^1)); 2.01, 2.02 (2*s*, Me(2^1)); 2.11, 2.12 (2*s*, Me(12^1)); 2.27 (*s*, Me(7^1)); 2.29–2.42 (*m*, H_B–C(20), CH_2(17^2)); 2.56–2.70 (*m*, CH_2(8^1), CH_2(17^1)); 2.88 (*m*, H_A–C(20)); 3.59–3.46 (*m*, 2 of 7 H, CH_2(8^2)); 3.61 (*s*, 3 of 7 H, MeOOC–C(17^2)); 3.62–3.68 (*m*, 2 of 7 H, CH_2(3^2)); 3.77 (*s*, MeOOC–C(13^2)); 3.93–4.08 (*m*, CH_2(10), H–C(1)); 4.54 (*m*, H–C(3^1)); 9.37 (*s*, H–C(5)). FAB-MS: 695.5 (8), 694.4 (40), 693.4 (88, $[M+H]^+$), 692.4 (27); 664.6 (13), 663.6 (25), 662.6 (15); 537.3 (40), 536.3 (100, $[M-$ring A$]^+$).

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[8]) ^{1}H-NMR (500 MHz, $CDCH_3$, 26°) showed additional signals at 4.85 (*d*) and 3.66 (*m*); the former is expected to be present in the spectrum of **10** in MeOH solution, but to be overlapped by the intense OH signal.

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